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DENITRIFICATION STUDIES ON SOME SOUTH AUSTRALIAN SOILS

by J. W. McGARITY *

Waite Agricultural Research Institute, Adelaide, South Australia

INTRODUCTION

Denitrification is now generally accepted as a potential biological process in soils in which aeration is restricted. Therefore it might be assumed that reduction and denitrification of nitrate leached from surface horizons could occur in deep subsoils where low oxygen tensions may exist during part of the year. This has been supported to some extent by evidence of evolution of nitrous oxide in the subsurface horizons of field soils [1], and the presence of denitrifying micro-organisms in the lower horizons of many of the great soil groups [6 11 12]. However, denitrification in anaerobic soil horizons is dependent not only on the presence of nitrate and nitrite as electron acceptors in the oxidative breakdown of organic matter by soil micro-organisms, but also on the content and nature of the decomposible organic matter itself [3 7].

Recently Bremner and Shaw [2], working with a range of soils of varying organic carbon content, were unable to detect denitrification under their experimental conditions when the levels of native soil organic carbon were less than 1 per cent. When glucose was added to these soils, however, denitrification proceeded rapidly. This observation suggests that the levels of soil organic carbon which occur in surface soils in sub-humid regions may be too low to provide sufficient energy material for active metabolism of denitrifying organisms even if all other conditions for the denitrification process are optimal. In addition, it would appear

* Present address: University of New England, Armidale, New South Wales, Australia.

that the deeper horizons of the soil profile with even lower soil organic carbon levels would be most unlikely sites for denitrification. Studies were therefore undertaken to determine the extent and nature of denitrification in soil samples from the genetic horizons of two sub-humid Australian great soil groups.

A modification of the manometric method proposed by Gilmour, Bollen and Damsky [5] was used to determine denitrification in the present studies. A description of the method is included and also data indicating its reliability. The effects of such substrate factors as water, nitrate, and glucose levels have been examined in the appraisal of the method.

MATERIALS

Soil samples from the red brown earth and black earth great soil groups [13] were collected at the Waite Institute from five sites with different histories of utilization.

The red-brown earth, (Urrbrae loam) at the Waite Institute is characterized by a red-brown, fine sandy loam to loam, soft granular surface soil, changing gradually to a red prismatic clay subsoil, with free calcium carbonate appearing at depths of 40 to 50 inches. Soil reaction in the surface is pH 5.6, rising gradually to pH 8.4 in the zone of free lime. The black earth (Claremont clay loam) has a dark-brown to black, granular to sub-angular blocky, clay loam surface soil, with a dark-brown, massive clay subsoil showing large columnar cracking and containing concretions and streaks of free lime. Reaction ranges from pH 7.0 at the surface, to pH 8.4, in the lime horizons which appear at a shallow depth.

A surface sample 0 to 1″ of red-brown earth under 14 years pasture of Wimmera rye grass and sub-clover was used in initial experiments. Samples were taken from shallow pits on May 11 and July 31, 1959. In the intervening period the content of NO_3-N fell from 60 ppm to 13 ppm. These samples contained 3.06 per cent and 3.18 per cent organic carbon respectively. The samples were dried in a forced-draft oven at 45°C. Aggregates of 2 to 5 mm size were obtained by seiving and stored in jars until required.

Three profiles of red-brown earth were sampled from the Waite Institute permanent-rotation plots established in 1927. The rotation treatments were continuous wheat, permanent pasture, and fallow-wheat-pasture-pasture at present in fallow (FWPP). Further details may be found in the Waite Institute Report [14]. The soils were sampled in 3″ layers to a depth of 12″, with the exception of permanent pasture where samples of 0–3″, 3–6″, and 6–12″ were collected. A Jarrett 4″ post-hole type soil auger was used to sample 6″ layers of soil from 12 to 48″. As the genetic horizons were diffuse it was not generally possible to determine the exact location of the horizon

boundaries. For this reason the 6″ layers may comprise whole or part of a complete genetic horizon. Precautions were taken in sampling to prevent microbiological contamination of soil samples.

The organic-carbon content at 0–3″ in permanent pasture sample was 2.63 per cent; in FWPP 2.12 per cent; and in continuous wheat, 1.65 per cent, reflecting the differences in utilization. Other analytical data are reported in the tables of results.

The black-earth profile under Wimmera rye grass sub-clover permanent pasture was sampled as above. A heavy $CaCO_3$ horizon commencing below 18″ became so impenetrable as to make sampling impossible below 36″. The organic-carbon content of the surface horizon was 5.24 per cent.

METHODS

Manometric method

The quantitative determination of small nitrogen losses from soils (less than 100 ppm) has usually depended on the determination of evolved gases by N^{15} tracer techniques [8 9 15], or on the difference between the initial total-nitrogen level and the final level in the soil [2]. The latter method can yield satisfactory results only when the naturally occurring field soil is subject to an intensive pretreatment of grinding and mixing to ensure uniform distribution of the total soil nitrogen, thereby eliminating sampling variation. This pretreatment must alter many of the physical, chemical, and microbial characteristics of the soil sample.

In the study reported here, denitrification in soils was followed using the Warburg manometric method. Other workers have indicated the possibility of using this method for soil denitrification studies under anaerobic conditions [5], and the results reported herein confirm the value of this approach. Apart from eliminating the need for extensive pretreatment of the soil, the manometric method affords a simple means of measuring changes in the rate of denitrification over comparatively short time intervals.

Respiration vessels of approximately 50 ml capacity were used. They contained a slightly off-centre inside well in which KOH solution was placed for the absorption of CO_2 (and H_2S). A vented sidearm permitted gassing of the vessels and their contents during the period of equilibration in the constant-temperature bath. Appropriate substrate additions of KNO_3 or glucose solutions were applied uniformly and dropwise using a hypodermic syringe to 10 g of air-dry soil aggregates contained in the respiration vessels. A measured quantity, usually 0.6 ml of 5 or 20 per cent KOH, was pipetted into the center well which contained a piece of folded filter paper.

The respiration vessels were closed with a tightly fitting rubber bung, the side-vent plug was opened, and the vessels were then placed in a vacuum desiccator. The vessels and their contents were evacuated to 1 mm (Hg) pressure and back flushed to room pressure with argon *. This procedure was repeated four times. In the final flushing with argon, the pressure was increased to slightly in excess of room pressure, the desiccator lid was removed, and the side-vent plugs immediately turned to the closed position. The respiration vessels were then attached to the manometers, placed in the constant-temperature bath (30°C), and gassed with purified argon for 30 minutes. This period sufficed for temperature equilibration, and the whole operation of adding substrates and gassing was completed in 90 minutes. The argon used for the final gassing was passed through a train of alkaline pyrogallol, sulphite-anthraquinone, KOH, and distilled water to absorb traces of oxygen. Check determinations using heat-sterilized soil, and pyrogallol and KOH reagents in the center wells of respiration vessels have shown that no uptake of gas attributable to O_2 can be detected manometrically using this evacuation and gassing procedure.

The duration of experiments varied from 144 to 400 hours and manometric readings were made at frequent intervals, generally every 4 hours. Evolution of gases other than CO_2 was indicated by the development of positive pressures in the respiration vessels. On the assumption that the gases measured were N_2 and N_2O, gas evolution has been reported as micrograms of N per gram of oven-dry soil.

Analytical methods

Chemical and physical properties of the soils were determined by the following procedures: Soil organic carbon by dry combustion [10]; total soil nitrogen by the modified Olsen digestion method [2]; and NO_2-N and NO_3-N on soil extracts by the α-napthylamine—sulphanilic acid and phenol-disulphonic acid methods respectively.

Saturation capacity was determined by measuring the water content of aggregates held at zero water tension for 24 hours on a sintered-glass Buchner funnel. For subsoil aggregates which broke down badly on wetting, the approximate water content at zero tension was estimated by multiplying the water content at 100 cm tension by a factor of 2. This factor was established from the water contents of non-disintegrating subsoil aggregates determined at the two tensions.

EXPERIMENTAL RESULTS

The relationship of gas evolution and soil-nitrogen loss

The efficacy of the manometric method for denitrification studies depends on the assumption that any positive pressure developed in the respiration vessels after all the CO_2 produced

* Commonwealth Industrial Gases argon containing 5 ppm oxygen.

by microbial metabolism has been absorbed by KOH is due to the evolution of either N_2 or N_2O. If N_2 and N_2O are the gases evolved, a close quantitative relationship should exist between gas evolution measured by the manometric method in the presence of KOH, and loss of NO_3-N and total soil nitrogen. Accordingly, the following experiment was conducted to determine the validity of this hypothesis.

Red-brown-earth aggregates containing 13 μg/g NO_3-N from the surface soil (0–1″) under 14-year pasture (July samples) were used in this experiment. KNO_3 equivalent to 101 μg N/g oven-dry soil (ods) was added to the air-dried soil in the respiration vessel and the water content adjusted to approximately 90 per cent of saturation capacity. Treatments numbered 1 to 5 were set up. After gassing and equilibration as described above, manometric readings were taken at 3- to 4-hourly intervals.

With Treatment 1, the respiration vessel was removed after manometric readings indicated that approximately half the theoretical denitrification of the nitrate had occurred. Determinations of NO_3-N, NO_2-N and total soil nitrogen were made on portions of the soil contained in the respiration vessel immediately after removal of the vessel from the manometer and bath. The remaining treatments were similarly analysed when a rapid fall in rate of gas evolution indicated near completion of denitrification, (Table 1). With Treatment 5, manometric readings were continued for a further 60 hours after completion of gas evolution, (Figure 1).

The level of initial total nitrogen was determined also on five replicates of aggregates to which 101 μg N/g as KNO_3 had been added. The results have been included in Table 1, and a nitrogen balance has been drawn up using average values.

The data show a close relationship of increasing total gas production coupled with an increasing loss of NO_3-N and total nitrogen. This same relationship of NO_3-N disappearance and gas production was found in all subsequent experiments. While traces of other gases such as H_2, CH_4, NH_3, H_2S may be evolved, there is little doubt that this method measures essentially the rate and course of conversion of NO_3-N and NO_2-N into nitrogenous gases.

TABLE 1

Nitrogen loss and gas evolution from Urrbrae loam using the manometric method						
Treatment	Final level in respiration vessel μg N/g					Initial total soil N †
	NO_2-N	NO_3-N	Total soil N *	Gaseous N **	total N	
1	20	20	2730	45	2775	2738
2	1	8	2726	103	2829	2779
3	1	10	2709	111	2820	2835
4	1	7	2710	119	2829	2837
5	1	22	2746	94	2840	2845
Average:						
Final	5	14	2724	94	2819	
Initial *	0	114	2807	0	2807	

* Includes NO_2-N and NO_3-N.

** Calculated from manometric readings.

† Determination on independent soil samples containing initial addition of 101 μg NO_3-N/g.

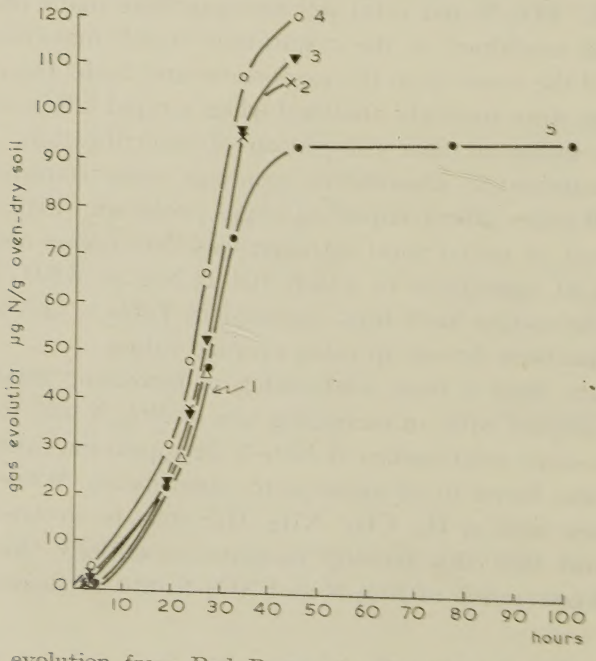

Fig. 1. Gas evolution from Red Brown Earth containing added NO_3-N. Numbered graphs refer to treatments in Table 1.

Factors influencing denitrification

(a) Water content. The effect on denitrification of varying soil water contents was determined using the manometric technique. Soil aggregates from the 14-year-pasture samples collected in May and containing 60 μg NO_3-N/g were used in these experiments.

Gas evolution was followed manometrically with 11 treatments containing 10 g of soil aggregates to which a further 103 μg NO_3-N/g of soil and varying quantities of water had been added. The center-well contained KOH, and the usual gassing procedure was followed. Water content, NO_2-N, and NO_3-N were measured at the end of each experiment. The final water content is reported, and represents a content lower by 2 to 4 per cent than the calculated initial level. Typical results are graphed in Figure 2.

The gas-evolution curves indicate an initial short lag period followed by a steady rate of gas evolution until the NO_3-N substrate is depleted. This pattern applies to all treatments except that with the lowest water content, (near wilting point). The uniformity of the pattern of the curves enables the denitrification

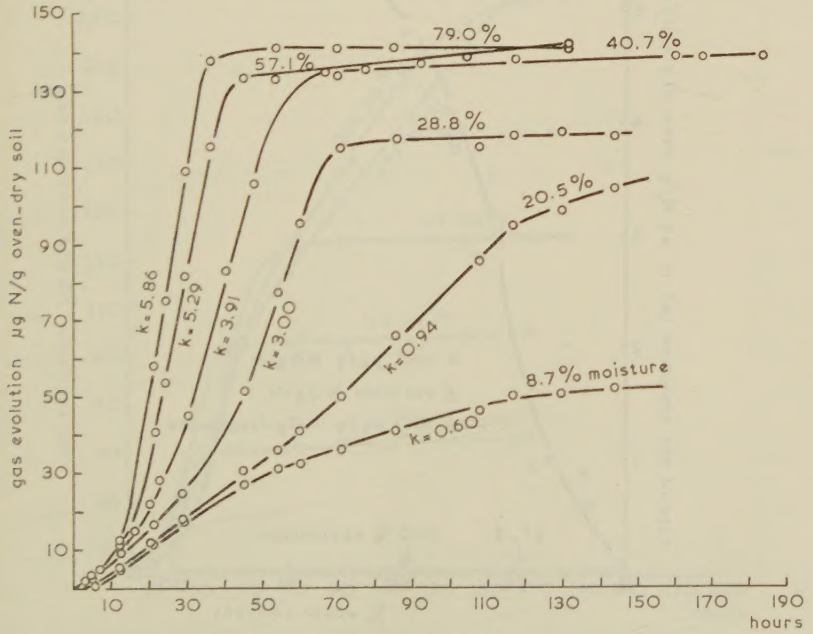

Fig. 2. Effect of soil water content on denitrification.

process to be characterized by the rate of gas evolution ($\bar{k}$) as μg N/g oven-dry soil per hour over the linear part of the curve. In this experiment the $\bar{k}$-values are also the maximum rates of gas evolution. The results under anaerobic conditions show that increased rate of denitrification is related to an increase in soil water content. The total denitrification also increases from the lower moisture range under these experimental conditions up to 40.7 per cent H_2O or approximately 80 per cent of saturation capacity.

Other experiments were performed on the same soil sample as used above. In one such experiment glucose was added and the water content was varied. In another experiment the level of nitrate addition was varied within a narrow range of soil water levels. These experiments are described below, but the results in relation to water content are considered here. Since the pattern of denitrification with this soil is characterized by the maximum

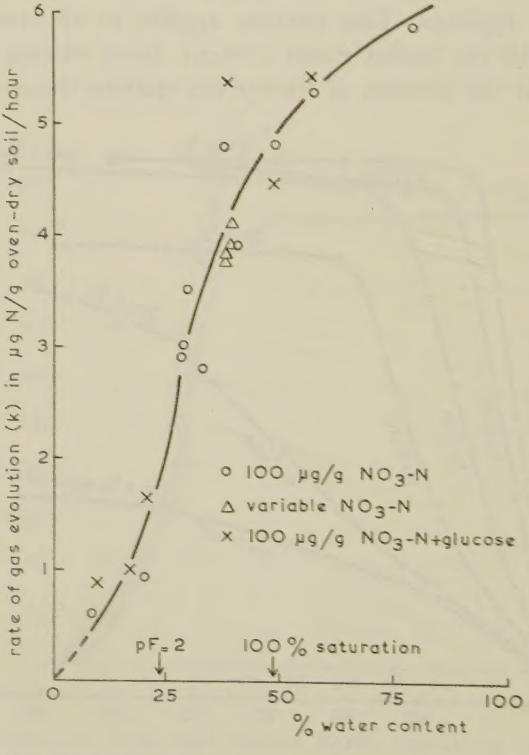

Fig. 3. The effect of water content on rate of denitrification.

rate of denitrification, this rate has been graphed against water content, (Figure 3).

The graph shows that under anaerobic conditions the rate of denitrification increases as the content of soil water increases within the range of wilting point to 165 per cent saturation capacity. It follows that for the purpose of comparative denitrification experiments, it is important to standardize the water content.

(b) Content of nitrate nitrogen. Red-brown-earth 14-year-pasture soil aggregates (0–1″) containing 60 μg N/g were used in an experiment to determine the effect of different contents of NO_3-N on denitrification. The soils were brought to approximately 85 per cent of saturation capacity with solutions of KNO_3 which added NO_3-N equivalent to 0, 52, 103, 206, and 412 μg N/g. Gassing and manometric procedure were as previously described.

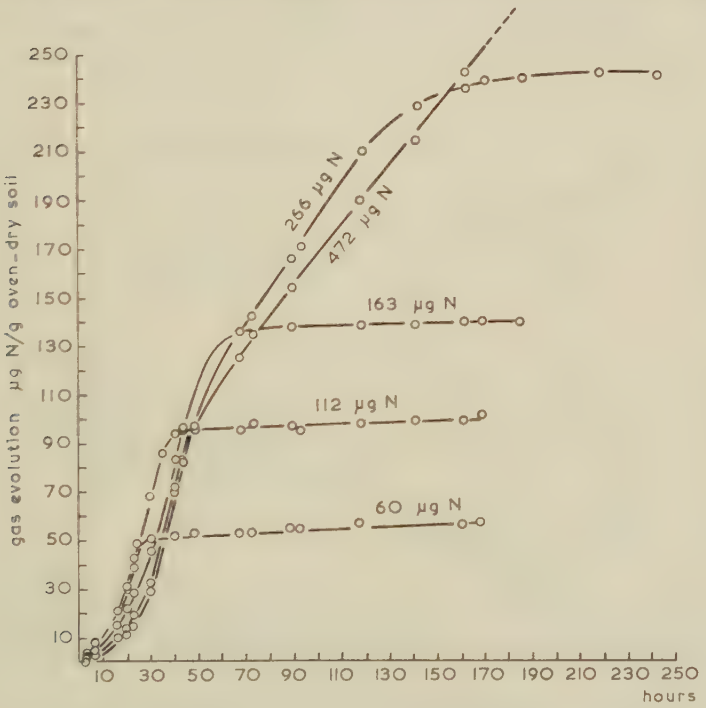

Fig. 4. The effect of initial nitrate content on denitrification.

The results indicate that between 60 μg N/g and 472 μg N/g the level of NO_3-N does not influence the maximum rate of denitrification (Figure 4). However, with levels above 112 μg N/g, a lag in the onset of the maximum rate is evident with increasing concentrations of NO_3-N. This lag does not alter the pattern of denitrification and in subsequent experiments 100 μg NO_3-N/g of soil was added as the usual substrate irrespective of the level of native soil NO_3-N.

(c) Effect of organic-matter addition. The importance of a high level of decomposible organic matter as a substrate to supply the energy needs of denitrifying micro-organisms is well known. As a preliminary study, denitrification was followed in four soils containing different quantities of native soil organic carbon.

The soils were red-brown-earth 0–3″ permanent pasture (2.63 % org. C), 0–3″ continuous wheat (1.65 % org. C), 0–3″ FWPP (2.12 % org. C), and 30–36″ permanent pasture (0.38 % org. C. NO_3-N equivalent to 100 μg N/g was added to each, and the water

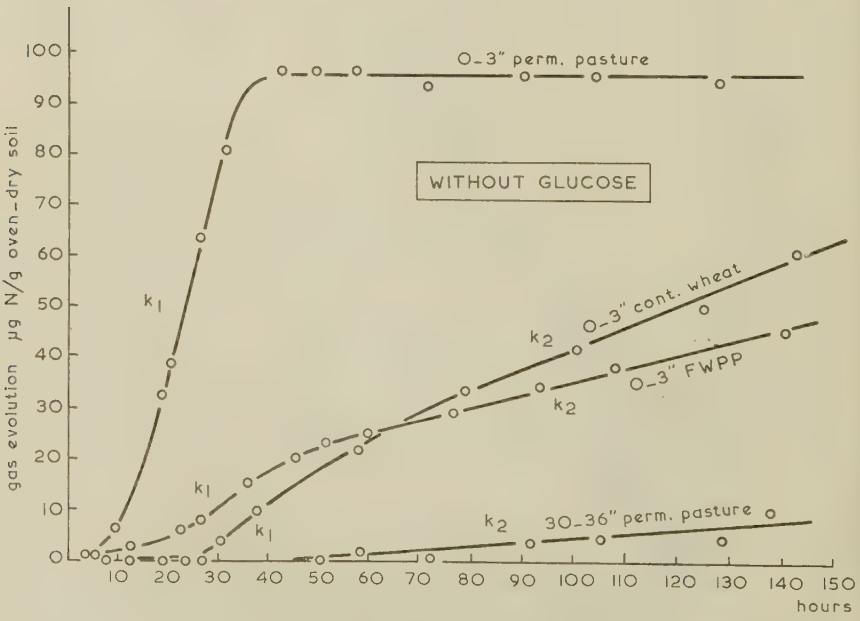

Fig. 5. Patterns of denitrification in 3 surface soils and 1 subsoil of red-brown earth without added glucose substrate.

content brought to 90 per cent saturation. Treatments without glucose and with 0.1 per cent glucose were set up.

The results of treatments without glucose indicate three distinct patterns of gas evolution, (Figure 5). The 0–3″ permanent pasture shows a short lag followed by rapid attainment of a steady and high rate of gas production, which eventually ceases as the NO_3-substrate is depleted. The second pattern, represented by the continuous-wheat and FWPP treatments, also shows a short lag followed by at least two different rates of gas production: An initial higher rate, k_1, followed by a lower rate, k_2. A third pattern is represented by the 30–36″ permanent pasture in which a long lag is followed by an extremely slow rate of production, k_2.

In the treatment in which glucose was added, all soils showed a more rapid rate of denitrification, (Figure 6). This increase in rate was least in the 0–3″ permanent pasture containing a high level of native organic matter. In the 0–3″ continuous wheat, 0–3″ FWPP, and 30–36″ permanent pasture samples, the pattern of the gas evolution was changed to a pattern similar to that of the 0–3″ permanent pasture. The length of the lag phase was also

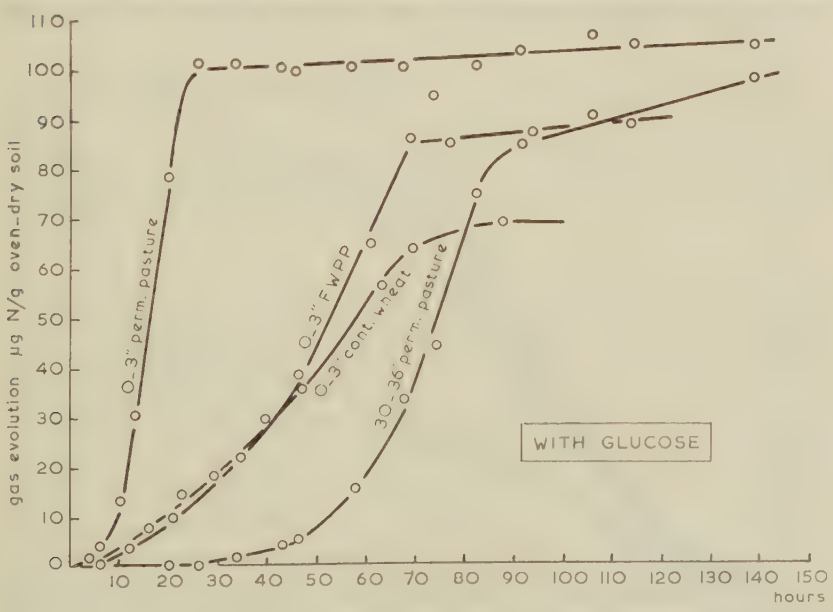

Fig. 6. Patterns of denitrification in 3 surface soils and 1 subsoil of red-brown earth with added glucose substrate.

shortened by the addition of glucose. These results indicate that the level of assimilable organic carbon is a major factor determining the pattern and maximum rate of gas evolution.

A further experiment was carried out to determine the pattern of response to low levels of glucose addition. Aggregates from the 0–3″ continuous-wheat rotation were placed in the respiration vessels, and a substrate of KNO_3 (100 μg N/g) and glucose (0, 0.005, 0.01, 0.05, 0.1, and 0.2 per cent) was added. The soils were brought to a water content of 90 per cent saturation capacity.

The addition of 0.01 per cent glucose (0.004 per cent carbon) is sufficient to change the rate of gas evolution quite markedly (Figure 7). With increasing amounts of glucose above the 0.05 per cent level, very little change in the rate and course of gas evolution occurs. This suggests that a limit to the rate of denitrification is imposed by factors other than the content of readily utilizable energy material.

This is also demonstrated by the results (Table 2) of a further experiment with 0–1″ permanent pasture (May), which contained a high level of organic carbon. Addition of glucose (0.1 per cent) at varying moisture levels did not stimulate any greatly increased

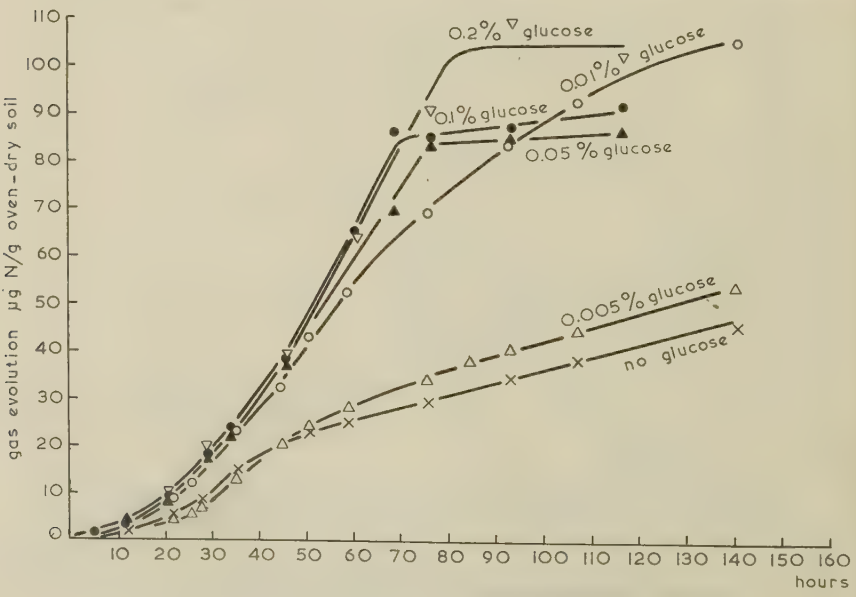

Fig. 7. The effect of glucose addition on denitrification.

rate of gas production. The patterns were of similar type to those depicted in Figure 2.

TABLE 2

Effect of water content and glucose on rate and quantity of nitrogenous-gas production

No Glucose			Glucose (0.1%)		
% H_2O	Rate (k) *	Total N gas **	% H_2O	Rate (k) *	Total N gas **
8.7	0.60	52	9.4	0.87	63
20.5	0.94	110	16.6	1.00	92
28.8	3.00	121	27.5	2.83	106
40.7	3.91	132	38.7	5.35	111
48.8	4.83	147	49.0	4.45	104
57.1	5.29	136	57.1	5.40	100

* μg N/g oven-dry soil per hour.

** μg N/g oven-dry soil after 150 hours or completion of gas evolution.

It is obvious that once a certain level of assimilable organic carbon is reached, then additional amounts do not increase the maximum rate of denitrification appreciably although it may prolong the period during which the maximum rate of denitrification is maintained. This is suggested by the shape of the curve for 0.01 per cent glucose in Figure 7, and also by the shape of the curves in Figure 4 of the highest levels of NO_3-N addition. In Table 2, the amount of gas produced is less with glucose than without. Apparently more NO_3-N is immobilized as microbial protein in the presence of glucose.

(d) Distribution of denitrifying organisms. To test for the presence of denitrifying organisms in the surface and subsoil, selected depth samples (Table 3) of red-brown-earth permanent pasture were placed in respiration vessels and the pattern of denitrification determined over 144 hours. One set of soils was supplied with glucose (0.1 per cent), and NO_3-N (100 μg/g), and the other set with NO_3-N (100 μg/g) only; all soils were brought to 90 per cent saturation.

It is evident that denitrifying organisms are present to the depth studied, although the longer lag period in denitrification for subsoils suggests that the numbers may be considerably lower than in the surface and sub-surface horizons. It is apparent from

TABLE 3

Denitrification in red-brown earth under permanent pasture								
Depth (inches)	Initial soil NO_3-N (μg/g)	Glucose substrate *	Gas evolution (μg N/g) at stated hours				Final soil	
			36	72	108	144	NO_2-N (μg/g)	NO_3-N (μg/g)
0–3	129	–	92	95	95	95	1	12
		+	101	100	104	104	0	13
3–6	120	–	17	41	56	67	12	37
		+	22	68	89	93	0	1
6–12	117	–	16	32	47	62	18	17
		+	40	89	91	88	0	2
18–21	103	–	19	36	54	68	18	21
		+	45	79	90	89	0	0
30–36	107	–	0	1	5	12	17	69
		+	2	40	90	98	0	0
42–48	113	–	1	0	3	13	9	76
		+	2	9	58	113	0	3

* – No glucose
\+ 0.1% glucose

the two sets of data that the lower rate of denitrification in the subsoils is the consequence of low availability of native organic substrates rather than the absence of organisms, for once given a source of decomposable organic matter, gas evolution increased rapidly.

Denitrification in the genetic horizons of red-brown earth and black earth

The foregoing studies indicate that denitrification of NO_3-N added to soil can be estimated by the manometric technique with an accuracy within the limits of soil and biological variation. Neither the differences in concentration of NO_3-N substrate produced when 100 μg NO_3-N/g soil is added to the level of native NO_3-N in the soil, nor slight variations in the water content near saturation capacity produces any marked effect on either the rate or the extent of denitrification. The major factor influencing

TABLE 4

Denitrification in the genetic horizons of black earth and red-brown earth

Depth (inches)	Genetic * horizon	% Org. carbon	Initial NO_3-N ** µg/g	Gas evolution † µg N/g				Final	
				36	72	108	144	NO_2-N µg/g	NO_3-N µg/g
Black earth – permanent pasture									
0–3	$A_{1.1}$	5.24	105	73	94	93	94	0	11
3–6	$A_{1.1}$	4.30	106	55	93	93	94	0	11
6–9	$A_{1.2}$	2.77	103	42	96	100	100	0	8
9–12	$A_{1.2}$	2.49	105	47	95	98	95	0	10
12–18	AB	1.61	105	40	91	101	100	0	10
18–24	BC_{ca}	0.83	104	24	41	51	61	19	21
24–30	BC_{ca}	0.52	102	9	24	45	52	22	26
30–36	BC_{ca}	0.38	102	4	10	13	15	32	43
Red-brown earth – permanent pasture									
0–3	$A_{1.1}$	2.63	129	92	95	95	95	1	12
3–6	$A_{1.1}$	2.26	120	17	41	56	67	12	37
6–12	$A_{1.2}$	1.82	117	16	32	47	62	18	17
12–18	AB	1.40	—	—	—	—	—	—	—
18–21	AB	1.05	103	19	36	54	68	18	21
21–24	B_1	0.42	103	4	11	12	14	8	62
24–30	B_1	0.27	104	2	9	10	13	12	70
30–36	$B_{2.1}$	0.33	107	0	1	5	12	17	69
36–42	$B_{2.2}$	0.40	—	—	—	—	—	—	—
42–48	$B_{2.2}$	0.36	113	1	0	3	13	9	76
Red-brown earth – continuous wheat									
0–3	A_p	1.65	116	9	30	45	61	6	7
3–6	A_p	1.06	131	6	16	25	27	12	47
6–9	A_p	0.53	102	6	15	19	23	16	59
9–12	$A_{1.2}$	0.41	104	8	15	21	21	10	53
12–18	AB	0.33	102	3	12	12	16	16	57
18–24	B_1	0.31	102	2	12	19	20	8	64
24–30	$B_{2.1}$	0.33	102	1	9	22	25	3	69
30–36	$B_{2.1}$	0.35	103	0	4	6	18	0	79
36–42	$B_{2.1}$	0.32	103	0	4	10	17	0	77
42–48	B_{ca}	0.26	103	1	4	10	15	3	68
Red brown earth – FWPP									
0–3	A_p	2.12	136	15	28	38	45	11	49
3–6	A_p	1.67	177	6	12	25	31	9	94
6–9	A_p	0.71	147	2	5	12	13	6	63
9–12	$A_{1.2}$	0.71	103	3	3	7	6	5	73
12–18	B_1	0.49	108	3	7	13	16	3	52
18–24	$B_{2.1}$	0.50	105	0	1	5	3	0	92
24–30	$B_{2.1}$	0.51	110	1	5	9	12	1	69
30–36	$B_{2.1}$	0.43	106	0	6	14	17	0	68
36–42	$B_{2.2}$	0.41	106	2	6	7	14	5	74
42–48	B_{ca}	0.38	104	1	3	2	6	15	42

* According to U.S.D.A. Soil Survey Manual Handbook 18; 1951.

** No NO_2-N in initial samples.

† Gas Evolution at various periods in hours.

denitrification under these experimental conditions is the level of assimilable organic carbon.

In the following series of experiments, the denitrifying ability of soil aggregates from the genetic horizons of the black earth under permanent pasture, and the red brown earths under permanent pasture, continuous wheat, and FWPP was determined by the described manometric technique. NO_3-N equivalent to 100 μg N/g was added to each soil in the respiration vessel and the water content was adjusted to 90 per cent saturation. As no organic matter was added, the denitrifying process was made completely dependent on the content of native soil organic matter as an energy-supplying substrate.

The gas evolution values at 36, 72, 108, and 144 hours have been obtained by interpolation from curves based on approximately four-hourly readings (Table 4). In some soils the gas evolution was still slowly continuing at the end of the 144-hour period.

Initial NO_3-N data have been included in Table 4 as well as the final NO_2-N and NO_3-N contents. The average recovery of NO_2-N, NO_3-N, and gaseous N, based on initial NO_3-N, was for the black-earth profile 97.8 per cent; and for the red-brown-earth permanent pasture 91.0 per cent, continuous wheat 87.5 per cent, and FWPP 74.3 per cent. The difference between the recovered nitrogen and initial NO_3-N may be ascribed to either assimilation of nitrogen by micro-organisms, or conversion in the pathway of denitrification to nitrogen compounds which have not been estimated, or both.

Of significance, however, is the finding that denitrification is possible in the subsoil B-horizon, even at depths of three to four feet, given conditions of anaerobiosis and a supply of NO_3-N. Although both initiation and rate of denitrification in soils from these depths is slow compared with the surface horizons, it is nevertheless a distinct and measurable process.

The relationship of denitrification to the genetic horizons is apparently a function of the organic carbon entity. This is shown in Figure 8, where the total denitrification at 144 hours is directly related to the level of total soil organic carbon. It should be noted that the horizontal portion of the graph for the black earth reflects the almost complete conversion of substrate NO_3-N addition to gas in horizons containing more than 1.61 per cent organic carbon.

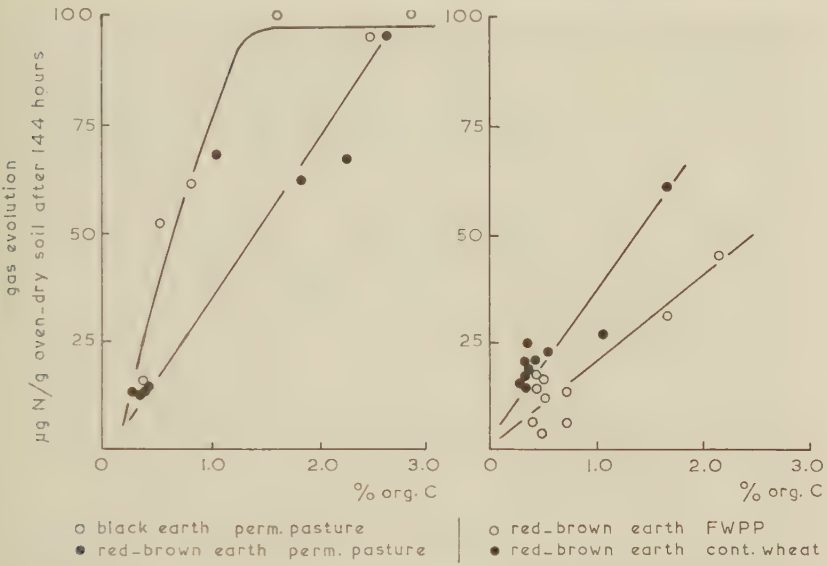

Fig. 8. The relationship of denitrification and native organic carbon content (in oven-dry soil) in profiles of 3 red-brown earths and 1 black earth.

The rate of evolution of gas differs between the profiles, and is greater per unit of native organic carbon for the black-earth permanent pasture, than for the red-brown-earth permanent pasture, (Figure 8). Of the red-brown earths, the FWPP rotation shows the lowest gas production per unit organic carbon. The reasons for these differences in rate of denitrification have not been investigated, but for the red-brown earths they may relate to the proportion of readily utilizable organic carbon in the total organic matter, which in turn is probably a reflexion of differences in the rotation treatments.

DISCUSSION AND CONCLUSIONS

Although various workers have suggested the possibility that gases such as H_2, CH_4, NH_3, H_2S may be evolved during denitrification experiments in closed anaerobic vessels [5 9], these gases have not been recorded even when total gas analyses of the vessel atmospheres were made by scan mass spectrometry [8 9]. The consistent relationship of NO_3-N disappearance to gas evolution

calculated as nitrogen, supports the view that nitrogenous gases are the only gases measured by the described manometric technique. It has not been possible to distinguish between N_2O and N_2 in the present work. It is noteworthy that the use of argon has effectively prevented anaerobic nitrogen fixation by free-living organisms, as shown by the stable level of gas pressure maintained in vessels after denitrification had ceased. The manometric technique reported here can therefore be regarded as a rapid and effective means of investigating many of the factors influencing denitrification in soils.

The effect of increasing soil water content on denitrification yielded similar data to those of other workers [2 9]. Bremner and Shaw [2] have shown that there is an increase in rate of denitrification in flasks open to air with increasing water contents from 50 per cent water-holding capacity to beyond saturation. They attributed this to a decrease in the rate of diffusion of oxygen into soil at higher moisture levels, and consequent increase in anaerobiosis. Some other explanation is needed for the results of the experiments reported here, (Figure 2). It would appear that the activity of the denitrifying organisms is controlled by the volume of water available for proliferation, and that the limit to increased rate of denitrification with increased water content is probably determined by complex nutritional factors.

The effect of readily utilizable organic carbon on denitrification in the red-brown earths is demonstrated by experiments in which glucose additions were made (Figure 7, Table 3 *etc.*).

From these experiments it appears that the utilizable organic carbon present in these soils determines to a large extent the patterns of gas evolution. The slow evolution of gaseous nitrogen from subsoil samples containing low levels of total organic carbon may therefore be attributed to a slow release of energy materials suitable for utilization by the denitrifying organisms. These utilizable materials could be derived from the breakdown of more complex soil organic matter by other groups of organisms. However, the greater part of the total denitrification in the initial 144 hours appears to result from use by the denitrifying organisms of the more decomposible energy material already present in the soil. The direct relationship of denitrification to total organic carbon in the soils examined suggests that the decomposible

fraction utilized by the denitrifying organisms must also be directly related to the total carbon content. Since denitrification is essentially a respiration process these results may be compared with a similar relationship linking total soil organic matter to respiration which has been found under aerobic conditions with the general soil microbial population [4].

The differences in rate of denitrification, and total denitrification, shown between profiles and between the various horizons of each soil profile may therefore be ascribed to differences in levels of utilizable organic carbon. It would appear also that soil organic carbon is more readily utilized in the black-earth profile than in the red-brown earth, and that organic matter in the permanent pasture and continuous wheat is more available for utilization than in FWPP.

This study indicates that the amount of organic carbon in the subsoils of the black earth and red-brown earths is far below the level which is needed to give high rates of denitrification, even over comparatively short time periods and with the favourable conditions of these experiments. Nevertheless a content of soil organic carbon as low as 0.3 per cent can still supply energy substrate to denitrifying organisms, thus producing measurable gas evolution. While the surface horizons have a much higher potential denitrifying activity, the subsoils also possess a potential activity which could lead to significant agronomic losses of nitrogen. The question remains, however, of the extent to which denitrification occurs in the field; the present study indicates that neither lack of organic carbon content nor lack of organisms should be factors limiting denitrification in these soils.

SUMMARY

A manometric technique based on a method proposed by Gilmour *et al.* [5] was shown satisfactory for the study of the rate and pattern of denitrification in soils under anaerobic conditions. Using this method increases in water content in the soil were found to increase the rate of denitrification. Additions of glucose markedly increased the rate of denitrification in soil with low content of native organic carbon but the increase was only slight in soils with high native levels. The content of NO_3-N in the range of 60 to 472 μg/g of soil did not affect the denitrification rate or pattern. Denitrification was not prevented by absence of micro-organisms in any of the surface or subsoils investigated.

Experiments with the genetic horizons of three red-brown earths and one black earth showed that denitrification of added NO_3-N took place in these soils to a depth of at least 36″, under conditions of anaerobiosis. Surface-soil samples containing the highest level of native soil organic carbon had the greatest denitrifying activity measured over a 144-hour period. The deep subsoil with low levels of organic carbon (0.3%) showed a much weaker denitrifying activity; nevertheless the potential activity in these horizons under field conditions could have significance in the soil nitrogen cycle. The total denitrification in 144 hours was directly related to the level of organic carbon in these experiments.

The denitrifying activity of red-brown earths from rotation plots was greatest in the order of permanent pasture, continuous wheat, fallow-wheat-pasture-pasture. Differences not only in total content of soil carbon, but also in the availability of the soil carbon as an energy substrate are considered to account for this order.

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METHODS LIKELY TO REDUCE THE GLASSHOUSE AREA NEEDED FOR EXPERIMENTS WITH THE TOMATO

by N. G. ALVEY

John Innes Institute, Bayfordbury, Hertford, Herts., England

INTRODUCTION

In a previous paper [1] the error obtained in glasshouse experiments at Bayfordbury to study effects of cultural treatments was discussed. The percentage standard errors of the total yields of tomato fruit after ten weeks of picking were similar to those obtained in glasshouse experiments elsewhere [3 4].

But it is important to record early yield as this is the most profitable part of the crop and some effects may not be distinguishable after 10 weeks of picking. The percentage standard errors are high in the earliest weeks of picking, and large areas under glass would be needed to accommodate the necessary replication.

There seemed two ways whereby this glasshouse area could be reduced. The first was to take some measurement on the young seedling from which the influence of a cultural treatment on its early yield could be predicted. Then it should only be necessary to fruit plants subjected to treatments which showed promise at an earlier stage of development when the seedlings occupied less area. Methods for predicting *total* yield have been used for maize [6] and wheat [14].

The other way was to find methods of reducing the number of plants required to conduct an experiment on early yield of stated sensitivity. Two factors were considered. The first was the effect of plot size on the percentage standard error.

The second was the effect on error variance of using F1-hybrid tomatoes. Reducing genetic variability may not reduce total

variability if homozygotes are more influenced by environmental causes of variability than heterozygotes. F1 hybrids have given a more uniform, but just as quantitative response to treatment as inbreds.[2] Dorofeenkov and Wallace[3] consider that this will only occur if the organism is naturally outbreeding. Lewis[4] presents results from experiments using inbred tomatoes as parents which throw doubt on the assumption that variability of F1 hybrids is related to the type of balance an organism has attained in its breeding system. Lewis measured flower number, which is presumably correlated with fruit number. Yield also depends on individual fruit weight, which is negatively correlated with fruit number (Williams[5]). Thus, it seemed worthwhile to see if the early yield of the F1 hybrid *Ware Crop* was more, or less uniform than that of one of its self-pollinating parents *'Potentate'*. Both varieties are widely used in commerce, and would be considered suitable material for the type of experiments discussed.

METHODS

Fresh weight, dry weight, and stem height of seedlings, grown for about six weeks after cotyledon expansion, were recorded. The date of opening of the first flower on an inflorescence was scored. This score was inversely related to earliness of flowering so that a precocious seedling would have a high score. The ripe fruit was picked once a week, and the early yield taken to be that for the first four weeks. Seedlings that were to be fruited were

Fig. 1 Showing planting plan, row spacing and orientation of the crop house

planted out in an uneven span glasshouse (51 × 32 feet) with its longer axis running east and west (Fig. 1). In such a house an experimental design with two restrictions was found suitable, and latin squares were used. An approximation to the Guernsey system of spacing tomato plants was employed *i.e.* 42 × 18 × 14 inches. The planting plan is illustrated in the figure. All seedlings were grown in John Innes composts until they were planted in the glasshouse beds.

EXPERIMENTAL

Seed of 'Potentate' was sown in JIS and JIP1 and the seedlings pricked-out into 3½"-diameter clay pots and soil blocks containing either JIP1 or JIP2. Half were pricked out at 2 days, and half pricked out at 7 days after cotyledon expansion. These four pairs of cultural treatments were chosen as likely to affect the seedlings at different stages of development. The stem heights were measured whilst seedlings were still in pots or soil blocks. The seedlings were then cut and weighed. At the same time other seedlings which had been subjected to the same cultural treatments were planted-out, and later their dates of flowering and early yield were recorded. The effects of the four pairs of treatments on these measurements, and the early yield are indicated by the figures in Table 1.

Stem height was highly correlated with dry weight in this experiment ($r = 0.94$ with 30 d.f.). Fresh weight has been compared

TABLE 1

Comparisons of the effects of certain cultural methods on height, fresh and dry weight, date of flowering, and early yield

	Seed compost		Pricked out		Potting compost		3½ inch pots	Soil blocks	S.E. of mean
	JIS	P1	2 days	7 days	P1	P2			
Seedlings in pots									
Stem height (cm)	6.5	6.6	7.2	5.9	6.7	6.4	7.0	6.1	± 0.09
Fresh weight (g)	11.0	10.6	12.2	9.4	11.2	10.4	11.6	10.0	± 0.17
Dry weight (g)	0.96	0.94	1.11	0.79	1.01	0.89	1.05	0.85	± 0.019
After planting out									
Date of flowering									
1st. inflorescence	4.5	4.8	6.6	2.7	4.5	4.8	5.1	4.2	± 0.23
2nd. inflorescence	5.7	5.8	7.1	4.4	5.3	6.2	5.6	5.9	± 0.21
3rd. inflorescence	6.5	6.4	7.0	5.9	6.0	6.9	6.1	6.8	± 0.12
4th. inflorescence	3.8	3.8	4.1	3.5	4.1	3.5	3.5	4.1	± 0.14
First month's crop (ounces)	32	30	34	28	29	33	29	32	± 0.94

with dry weight in other experiments also, and the following correlation coefficients found:

$r = 0.94$ with 22 d.f.
0.99 with 10 d.f.
0.98 with 22 d.f.
0.97 with 30 d.f.

In other experiments seedlings were potted on into 4½″-diameter clay pots to simulate the effect of planting-out in a relatively small area. Up to flowering of the second inflorescence the results were similar to those obtained when the seedlings were planted out in the glasshouse beds (Table 2). Measurements were not made after this stage of development as the seedlings needed feeding with the consequent risk of increasing error by differences in the quantities of nutrients supplied to each pot. Also the plants would need spacing and would cover almost as large an area as if planted-out.

TABLE 2

Comparisons of the effects of certain cultural treatments on fresh weight, date of flowering, and early yield

	Pricked out		Potting compost		3½ inch pots	Soil blocks	S.E. of mean
	2 days	7 days	P1	P2			
Seedlings in 3½ inch pots							
Fresh weight (g)	15.9	11.6	14.0	13.5	14.8	12.7	± 0.24
Seedlings in 4½ inch pots							
Date of flowering							
1st inflorescence	4.8	2.5	3.8	3.5	4.0	3.3	± 0.15
2nd inflorescence	5.4	3.1	3.9	4.6	4.1	4.4	± 0.17
After planting out							
First month's crop (ounces)	29	17	19	27	20	26	± 0.96

Comparisons of the effects of some other cultural treatments on fresh weight, date of flowering and early yield were made (Table 3). The fresh weights of plants grown at different times of year in JIP1 and JIP2 are shown in Table 4.

The calculation of percentage standard errors is not appropriate to experiments where flowering date is recorded, as the means are arbitrary figures depending on the method of scoring. Direct use can

TABLE 3

Comparisons of the effects of certain cultural treatments on fresh weight, date of flowering, and early yield

Cultural treatment	Fresh weight of seedlings in 3½ inch pots, g	Date of flowering		First month's crop, ounces
		First inflorescence	Second inflorescence	
Supplementary illumination in propagating house				
Illuminated	13.4 ± 0.23	11.7 ± 0.39	11.5 ± 0.35	36 ± 1.3
Control	4.5	1.9	1.5	19
Temperature in propagating house				
High	12.2 ± 0.31	6.4 ± 0.24	10.8 ± 0.31	26 ± 1.1
Low	7.1	2.0	3.8	24
Temperature in cropping house				
Soil				
High		5.0 ± 0.32	5.5 ± 0.28	36 ± 1.7
Low		5.8	5.7	40
Air				
High		6.9 ± 0.32	8.3 ± 0.28	44 ± 1.7
Low		3.9	2.9	32
Use of synthetic soil conditioner				
Conditioned	8.6 ± 0.25	6.9 ± 0.23	5.7 ± 0.20	27 ± 1.2
Control	8.7	6.6	5.6	26

TABLE 4

A comparison of the effects of two potting composts on fresh weight (g) of tomato seedlings at different times of year

Period of growth	Mean fresh wt. per seedling		Standard error of mean
	JIP1	JIP2	
22nd February–27th March	11.04	10.83	± 0.24
6th–29th May	10.97	12.35	± 0.31
26th May–22nd June	7.08	10.13	± 1.20
1st–25th July	12.01	13.42	± 0.19
30th August–25th September	9.17	10.37	± 0.26
20th October–4th December	5.67	4.60	± 0.21

be made of error variances or standard errors to compare variabilities of different experiments. The variabilities of flowering in pots,

and in the glasshouse beds of different varieties and of different inflorescences are compared in Table 5.

TABLE 5

Comparisons of the variability of earliness of flowering				
	Error variance per plant	Degrees of freedom	Variance Ratio	Probability
Glasshouse beds	6.58	794	1.72	< 0.001
Pots	3.83	299		
Potentate	6.09	511	1.20	0.05–0.01
Ware Cross	5.08	511		
Potentate	5.92	45	1.09	> 0.05
Ailsa Craig	5.43	45		
Potentate	5.92	45	1.08	> 0.05
Best of All	5.49	45		
First inflorescence	6.08	375	1.16	> 0.05
Second inflorescence	5.18	375		
Third inflorescence	3.81	375	1.36	0.01–0.001

TABLE 6

Average weight of fruit (ounces) per plant picked from first four inflorescences				
Week of picking	1st infl.	2nd infl.	3rd infl.	4th infl.
1	2.1	—	—	—
2	7.0	2.1	—	—
3	4.2	5.7	1.0	—
4	1.5	3.0	3.0	1.0

The weight of fruit picked in the first four weeks from each inflorescence was recorded (Table 6).

To find how plot size and the use of F1 hybrids would affect the error variance associated with early yield, an experiment was arranged as six 8 × 8 latin squares in the glasshouse beds. Three of the latin squares were assigned at random to 'Potentate' and three to 'Ware Cross'. Plots containing one plant were used, and the residuals were calculated for each plot. From these residuals the percentage standard errors that would have been obtained using plots containing different numbers of plants were estimated. The average percentage standard errors for the various plot sizes are shown in Table 7. The single rows referred to run N and S and the double rows are a pair of these spaced 42" apart (see the Figure). Smith [1] has shown that the regression of the logarithms of standard

error on the size of plot is substantially linear. The observations on which Table 4 is based were not fully independent but the regression coefficients were calculated to give some idea of the relationship between log percentage standard error and log plot size. Each

TABLE 7

Average percentage standard errors for different plot sizes				
Plot size	Single rows		Double rows	
	D.f. per latin square	Average percentage s.e. per plot	D.f. per latin square	Average percentage s.e. per plot
One plant	49	28.7	—	—
Two plants	21	22.5	21	19.3
Four plants	7	17.1	9	15.1
Eight plants	—	—	3	10.3

observation was weighted by the relevant degrees of freedom. The values of the regression coefficients were: *Single rows* – 0.41, *double rows* – 0.48.

Table 8 shows the percentage standard errors of other experiments on early yield where different plot sizes were used. The regression coefficient, calculated in the same way, was 0.42.

The variability of early yield of the two varieties in this and in two other experiments, is compared in Table 9. In one case, the figures showing the variability of 'E.S.1' the other parent of 'Ware

TABLE 8

Percentage standard errors of experiments on the early yield of tomato 'Potentate'		
No. of plants per plot	Percentage standard error	Degrees of freedom
1	33.2	42
1	33.6	42
3	24.1	42
3	17.8	42
5	14.8	55
5	26.5	49
5	19.0	21
5	18.0	21
5	17.2	45
6	13.9	21
6	12.8	9
6	17.2	9
10	8.5	14

Cross', have also been included. No significant differences could be found between the mean percentage standard errors of the three varieties using the *t* test.

In these experiments on early yield differences in yield due to the original position of the seedlings on the propagating bench were calculated, and found to be insignificant.

TABLE 9

Comparing the variability of early yield of the F1 hybrid 'Ware Cross' with that of its parents

	Variance	Degrees of freedom	Variance ratio	Probability	Mean yield per plant (in ounces)	Standard error, %
Potentate	71	126	1.76	0.01–0.001	29	29 ± 2.3
Ware Cross	125	126			40	27
Potentate	79	14	1.38	> 0.05	39	22 (no replication)
Ware Cross	108	6			54	19
Potentate	42	24	1.21	> 0.05	22	29 ± 3.6
Ware Cross	51	24			29	25
E.S.1	32	24	1.59	> 0.05	26	22

DISCUSSION

Early yield includes fruit picked from the third and fourth inflorescences (Table 6). Thus, it should be possible to predict the effect on early yield of many cultural treatments from measurements taken up to anthesis of the fourth inflorescence. But it is not convenient to take measurements after anthesis of the second inflorescence as tomato seedlings would then occupy almost as large an area as they would if planted out for cropping. However, the effects of a number of treatments on early yield can be predicted from measurements taken at, or before, anthesis of the second inflorescence (Tables 1, 2 and 3). How early a reliable measurement can be taken depends on the stage of development at which the treatment is likely to influence the seedling. In certain cases *e.g.* the nutrient status of the potting medium, or the use of soil blocks, some, or all, of the early measurements can be misleading. The effect of the potting medium, on fresh weight is also influenced by the season when the seedlings are grown (Table 4).

Some treatments affect the manner, rather than the rate, of seedling development. For example, a high propagating temperature

accelerates growth, but also affects the position and size of an inflorescence [2]. Thus, seedlings grown at a high temperature in the propagating house can have greater fresh weight and flower earlier than those grown at a low temperature, yet their early yield is not significantly heavier (Table 3).

If it is considered reliable to take measurements on month old seedlings, fresh weight and possibly stem height give as trustworthy a prediction as dry weight, under conditions at Bayfordbury [7]. Ross [10] has found that dry weight was highly correlated with functions of stem height.

Variability of flowering earliness is lower when seedlings are grown in pots than when they are grown in the glasshouse beds (Table 5). This is probably because the compost in the pots is more homogenous than the soil in the beds and because each pot plant has a separate root run which will not interfere with that of its neighbour.

Earliness of flowering of the inbred tomato varieties 'Potentate', 'Best of All', and 'Ailsa Craig' seems more variable than that of the F1 hybrid 'Ware Cross' (Table 5). Similar results have been obtained with other F1-hybrid tomatoes (Lewis [8], Williams [13]).

There is also a tendency for earliness of flowering on the bottom inflorescences to be more variable than that on those higher up the stem. The higher inflorescences will generally flower as the declination of the sun, and the duration and intensity of its radiation, increase. These conditions are likely to produce a more uniform flowering response.

The most reliable measurement taken on tomato seedlings in small pots for predicting early yield is the one taken at the latest possible stage of development *i.e.* at anthesis of the second inflorescence.

When seedlings are to be fruited, the use of small, rather than large plots is not likely to decrease considerably the number of plants required to carry out an experiment of stated sensitivity. The values for the regression coefficients were near 0.5, a value which would apply if the units in each plot were uncorrelated. Even in this situation, however, one will enter the table of F with higher degrees of freedom for N2 by using low, rather than high, numbers of plants per plot. With the layout shown in Fig. 1, it might be an advantage to arrange plots across double rows, as the regression coefficient for plots so arranged was higher than that for plots arranged along single rows.

Although it was not possible to detect differences between the percentage standard errors of early yield of 'Ware Cross' and 'Potentate', the figures in Table 9 do make a consistent pattern. The variance of early yield of 'Ware Cross' is greater than that of 'Potentate' – in one case significantly greater – whereas the percentage standard error is smaller. In the experiment where the early yield of the other parent, 'E.S. 1' was also recorded, the percentage standard error of 'Ware Cross' was intermediate between that of its parents. From data obtained with other tomato hybrids Williams (unpublished) has postulated that, whilst variability of flowering date is inherited as a genetic character with low variability dominant, variability of early yield is intermediate between that of the parents. In these circumstances the use of an inbred variety with low variability should prove more effective in reducing the number of plants required for an experiment on early yield than the use of an F1 hybrid.

From the results given in this paper it appears that there is scope for reducing the number of experiments that need be conducted on the early yield of tomato, by eliminating unpromising cultural treatments at an earlier stage of development than fruiting. If it is necessary to carry out an experiment on early yield then high relative variability must be catered for. There seems little prospect of reducing it considerably, judging by the results in this and previous papers [1 7].

SUMMARY

Methods for reducing the area under glass needed for experiments with tomato were studied.

The effects of some cultural treatments in the propagating house on early yield can be predicted from measurements taken while the seedlings are occupying a relatively tiny area in small pots. In these cases, treatments which do not prove superior to the controls can be eliminated without studying their effect on fruiting plants occupying a relatively large area.

If measurements on month old seedlings are considered reliable, then fresh weight and possibly stem height give as trustworthy a prediction of early yield as dry weight.

If date of flowering is to be recorded then more uniform results are obtained by growing plants in pots rather than in glasshouse beds. Variability of the bottom inflorescences is greater than that of those higher up the stem.

The latest stage of development at which measurements can conveniently

be taken whilst the seedlings are still occupying a small area is the flowering of the second inflorescence. Measurements taken at this stage will give a reliable prediction of early yield with a wider range of cultural treatments than measurements taken at an earlier stage of development.

Where it is necessary to study early yield, the number of plants required to conduct an experiment of stated sensitivity is not likely to be much reduced by using small, rather than large, plots. This applies to plots containing up to ten plants which probably approaches the upper limit considering the glasshouse area normally available for such experiments.

It is likely that the percentage standard errors of experiments on early yield will be smaller when using an inbred variety of low variability than when using an F1 hybrid.

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EFFLUX AND RETENTION OF FOLIAR APPLIED PHOSPHORUS-32 AND SULFUR-35 BY INTACT BEAN ROOTS, AND THE INFLUENCE OF VARIOUS AMBIENT IONS ON THIS RELATIONSHIP *

by FRED H. EMMERT

Storrs Experiment Station, University of Connecticut, Storrs, Conn.

INTRODUCTION

Considerable evidence exists to show that passage of ions from plant roots to the external medium does occur. Colby [7] noted significant increases in electrical conductivity of solutions in which young prune trees were grown. Chromatographic tests by Frank [12] indicated nitrogen in the form of amino acids was lost from maize and asparagus roots to water cultures. Luttkus and Bötticher [18] found root loss of potassium to reach 15 to 30 per cent of the total plant content, and was especially rapid in dark periods. Increased root efflux of potassium during darkness was also noted by Andel *et al.* [2]. Burd [6] found loss of potassium and nitrogen from barley plants to coincide with stage of plant development, while Achromeiko [1] actually set up plant categories on the basis of root efflux. Phosphorus loss amounting to 4 to 25 per cent of original plant content was reported for corn by Fedorovskii [11]. Moore [19], Hevesey [13], and Brewer and Bramley [4] also noted loss of phosphorus from roots of various plants.

Although evidence of ion passage from roots to the milieu is impressive, not much is actually known of the mechanics involved in such transfer or forces which influence it. Greatest progress in this area of inquiry has occurred where isolated cells and tissues instead of whole plants were studied. Results have led to concepts

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of "free space" in tissues, and indicate that efflux involves a complexity of forces including free diffusion, ion exchange, and Donnan equilibria [5 8 10].

Current tests were conducted to learn more about the quantitative aspects of efflux from intact plant roots, and of possible influences of ion species in the recipient solution on the process. Tests involved foliar introduction of radiophosphorus or radiosulfur to bean plants, and measurement of loss of isotope from roots to various single salt solutions. Foliar rather than the more common root introduction techniques were favored to prevent contamination of root surface with isotope. Comparisons were made of fraction lost as opposed to the fraction retained by the root.

MATERIALS AND METHODS

Bean seeds (*Phaseolus vulgaris* var. Wade) were germinated and grown in a controlled-temperature bath according to techniques described elsewhere [9]. When stems were approximately 8 cm long, plants were removed to a controlled-environment facility and placed in 2 liters aerated complete nutrient solution. Isotope application was made following 4 days of growth in the facility. Approximately 20 μc of carrier-free P^{32} or 80 μc carrier-free S^{35} were deposited at mid-point of the main vein of one primary leaf of each plant in a 150 or 300 λ volume, respectively. Higher S^{35} than P^{32} activities were employed because of differences in MEV values between isotopes. The isotope was allowed to enter and become distributed throughout the plants for 2 days, after which time loss of material from roots to various single salt solutions was determined.

Immediately prior to the loss study, the leaf to which the isotope was originally applied was severed at the petiole and discarded. Microscopic flakes of radioactive tissue eminating from the area of isotope application were found to constitute a serious source of contamination. Plants were next moved to a shielded plastic holder, and roots placed in a beaker containing 100 ml of the old growth solution. The old solution was next drained and replaced with 100 ml of treatment solution. This change served as base time for the loss period. Fifty minutes from base time plants were removed from the treatment solution, roots were severed at the hypocotyl and prepared for counting. Loss solutions were filtered and counted. Adoption of a 50 minute loss period as standard procedure was based on time-course studies described later.

Following the loss period, roots of plants treated with P^{35} were ashed in a crucible for 24 hours at 450°C. The ash was taken up on 10% nitric acid, made to 100 ml with demineralized water, and a 10-ml aliquot evaporated in a planchet for counting.

Because of the volatile nature of sulfur at high temperatures, ashing

procedures were not undertaken with S^{35} treated plants. Roots instead were extracted 3 minutes in 100 ml sodium acetate buffer (pH 4.8) in a Waring blendor. Ten ml of the filtered extract was evaporated in a planchet for counting.

Macro-element composition (as milliatoms/l) of the complete nutrient solution used for starting the plants was N = 12, P = 1, K = 5, Ca = 3, Mg = 2 and S = 2. A full compliment of essential micro-elements was also provided. Root loss was studied to single salt preparations of the chloride, sulfate, and nitrate forms of potassium, magnesium, and ammonium. All possible combinations of these ions were employed at 0.5 and 6.0 me/l levels.

Radioactivity of all samples was determined in a gas-flow counter equipped with an ultra-thin end window (150 $\mu g/cm^2$). Counts were corrected for decay and background. No corrections were possible or believed necessary for geometry, self absorption, and backscatter.

All tests were designed to be susceptible to statistical evaluation. Each treatment was replicated with 6 plants, and accrued results were processed according to conventional analysis of variance procedures. Level of significance in all cases was set at the 5 per cent confidence interval.

RESULTS

Time-course study

Previous work indicated loss of P^{32} from roots follows an exponential pattern with time, and can roughly be divided into an initial period of rapid loss followed by a sustained period of slow loss. Dainty and Hope [8] and Leggett and Epstein [17] formed similar conclusions with I^{131} and S^{35}, respectively. It was decided to restrict attention in the current tests to the period of rapid loss, or what also may be called "short-term" loss. Extrapolation of earlier data with P^{32} indicated "short-term" loss was completed after 50 minutes under present experimental conditions [9].

In order to establish more clearly the exact boundaries of "short-term" loss, a 70-minute time-course experiment was undertaken with both P^{32} and S^{35}. Techniques of plant growth and isotope application were similar to those described. Two days after P^{32} or S^{35} application, plant roots were placed in individual glass tubes (10.0 × 2.5 cm) which were provided with drainage and aeration ports, and which contained 25 ml of the old growth solution. The old solution was drained and replaced with 25 ml of a 6.0 me/l KCl solution. Solutions were again drained and replaced at 5, 15, 30, and 50 minute intervals, with the final drainage occurring at the 70 minute mark. Roots and solutions were processed in the manner described. Results are given in Figure 1.

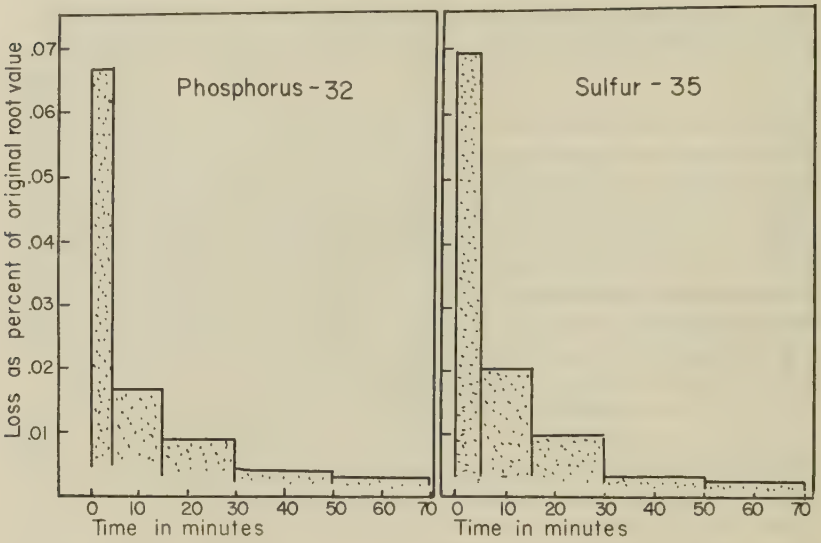

Fig. 1. Root efflux of P^{32} and S^{35} over a 70-minute period. Results are calculated as per cent of original root content lost in each minute of the test.

Time gradient of both P^{32} and S^{35} loss followed the expected exponential pattern. Approximately 0.07 per cent of isotope originally in the root was lost each minute early in the test, but this figure diminished to less than 0.01 per cent with time. The curve appeared to approach a steady state condition in the neighbourhood of the 50 minute mark, depicting the end of "short-term" loss.

On the basis of these findings, a 50-minute loss period was adopted as standard procedure in tests which follow.

"Short-term" loss to various nutrient environments

Quantitative relationships between μc isotope applied to the foliage, found in the root, and lost to the milieu, are given in Table 1. Only findings obtained with the potassium solution series are listed since they are representative of the whole. Large differences of logarithmic proportions were evident in activities between isotope applied, in the root, and finally lost. In very approximate terms, of every 1×10^6 P^{32} or S^{35} atoms placed on the foliage, only in the neighbourhood of 1×10^4 atoms were finally located in the root, and less than 1×10^2 atoms passed to the treatment solution (Root content of S^{35} represents the extractable fraction, and such figures

TABLE 1

Microcuries of isotope applied to bean foliage, located in the root, and lost to various potassium solutions

Salt	μc applied per plant *	μc in root before loss * †	μc lost by root	μc retained by root * ‡
Phosphorus-32				
KCl 0.5 me/l . .	19.7	0.1911	.0006	0.1905
6.0 me/l . .	19.7	0.2028	.0009	0.2019
KNO_3 0.5 me/l	19.7	0.1699	.0007	0.1692
6.0 me/l . .	19.7	0.1849	.0009	0.1840
K_2SO_4 0.5 me/l . .	10.6	0.0925	.0005	0.0920
6.0 me/l . .	10.6	0.1036	.0008	0.1028
Sulfur-35				
KCl 0.5 me/l . .	88.0	1.5845	.0085	1.5760
6.0 me/l . .	88.0	1.6634	.0103	1.6531
KNO_3 0.5 me/l . .	88.0	1.3940	.0078	1.3862
6.0 me/l . .	88.0	1.3944	.0087	1.3857
K_2SO_4 0.5 me/l . .	88.0	1.3819	.0088	1.3731
6.0 me/l . .	88.0	1.5895	.0090	1.5805

* Curie strength of initial plant applications as well as conversion of cpm data to microcuries were based on activity levels of stock as determined by the Oak Ridge National Laboratories.

† μc lost + μc retained by root.

‡ Values in the case of S^{35} for extractable fraction.

may be somewhat below true total values). Original activity was thus progressively reduced by factors of the order 1×10^{-2} and 1×10^{-4}, respectively. The approximations given here for the potassium solution series poignantly illustrate that only a small relative proportion of the total number of radioions considered participated in "short-term" loss.

This point is emphasized again in Table 2 where isotope loss was calculated as per cent of original root content. Values were below 1 per cent in all cases but one (P^{32} loss to magnesium solutions).

Although root loss involved relatively few of the applied or absorbed atoms, data in Table 1 show that such loss was conditioned by (a) species of ions in the ambient medium (b) level of concentration of such ions, and (c) the particular radioisotope studied. Regarding ion species effect, greatest exit of P^{32} occurred to Mg^{++},

TABLE 2

Loss of P^{32} and S^{35} from bean roots calculated as per cent of initial root content. Values were compiled for individual ions of the single salt recipient solutions

Ion concentration	Phosphorus-32			Sulfur-35		
	0.5 me/l	6.0 me/l	LSD $P = 0.05$	0.5 me/l	6.0 me/l	LSD $P = 0.05$
Cations						
K^+	0.40	0.57	0.13	0.59	0.62	n.s.
NH_4^+	0.46	0.76	0.23	0.66	0.75	n.s.
Mg^{++}	1.07	1.36	0.19	0.72	0.46	0.18
Anions						
NO_3^-	0.69	0.84	n.s.	0.59	0.55	n.s.
Cl^-	0.63	0.91	0.18	0.70	0.67	n.s.
SO_4^{--}	0.66	0.98	0.21	0.68	0.61	n.s.
LSD $P = 0.05$	0.14	0.17		n.s.	n.s.	

n.s. = not statistically significant at $P = 0.05$

and least to K^+ and NH_4^+. All anion values fell between these extremes. Ambient ion influences on P^{32} loss thus followed the sequence $Mg^{++} > Cl^-$, NO_3^-, $SO_4^{--} > K^+$, NH_4^+ (divalent cation > mono- and divalent anions > monovalent cations). This relationship held at both concentration levels and differences were statistically significant. Root loss of S^{35} on the other hand, was not differentially altered by any of the ambient ions at either concentration level.

Concentration of the external solutions also conditioned root loss. In the case of P^{32}, greater loss occurred to the high (6.0 me/l) than to the low (0.5 me/l) concentrations solution. This relationship was manifested for all solutions, and in most cases attained statistical significance. On the other hand, no statistically significant concentration effect was noted on S^{35} loss except to the magnesium solutions. Here greatest loss occurred to the low concentration solutions (contrary to the P^{32} data).

DISCUSSION

Both P^{32} and S^{35} were found to pass from bean roots to surrounding solutions under all experimental conditions. Quantitative aspects of loss were small, however, compared to amounts of radioions applied to the foliage and to those held by the roots. In most

cases, less than 1 per cent of what was in the root was lost. These findings in themselves discourage consideration of "short-term" root loss as a serious force affecting plant composition under present experimental circumstances. Care must be taken, however, not to project these conclusions to other than current conditions ("short-term" loss of foliar-applied isotopes to true solutions). Aggregate loss over an extended time period may, for instance, provide different answers. Loss to a medium abundant in electrically charged surfaces may also differ from that to a true solution. Jenny [14 15] found that barley roots held potassium tenaciously against salt solutions and water, but lost significant amounts of this element to variously charged systems.

The importance of careful consideration of experimental methodology in loss studies is illustrated in Table 3. Foliar applications of

TABLE 3

Effect of method of isotope introduction and of electrical properties of root medium on root loss of P^{32}. Values were calculated as per cent of initial root activity

Electrical properties of root medium	Foliar introduction	Root introduction †	LSD $P = 0.05$
Resin *	0.96 **	18.34 **	3.82
H_2O	0.54	16.19	4.04
LSD $P = 0.05$.	0.32	1.05	

* 4.0 grams Cl^--saturated Dowex 1-× 4 in 100 ml H_2O.

** Counts made after resin was filtered, dried, and ashed. Activity of H_2O phase also determined and added to that of resin.

† Roots were rinsed quickly in two H_2O baths and blotted thoroughly before being placed in loss solution.

P^{32} were made to one set of bean plants in the outlined fashion, while the isotope was added to growth solutions of another set of plants. Root loss of radioions to water and to a chloride-charged anion resin (Dowex 1-x4) was noted. Greatest loss clearly occurred where root introduction was practiced, and to the anion resin.

Although exit of isotopes from roots was of low order, the process was nevertheless conditioned by composition of the surrounding medium. An outstanding example in this respect was the expedition of P^{32} exit by magnesium. Greatest root loss in the entire study occurred for this combination. Such findings are of particular interest in light of considerable evidence that magnesium facilitates

phosphorus uptake (reviewed by Beeson [3]). Magnesium has also been suggested as a phosphorus carrier (Truog *et al.* [21], Mulder [20], Krachenberger and Peterson [16], and Zimmerman [23]). Magnesium has further been identified with phospholipid synthesis [23] as well as activation of phosphorylating enzymes [22]. It is difficult to reconcile evidence of a strong and positive influence of magnesium on incorporation of phosphorus in the plant system with current data showing enhanced loss of phosphorus from roots where magnesium is present.

Other ions as well as magnesium conditioned root exit of P^{32}. When arranged in order of decreasing influence, these ions formed the sequence: divalent cation > mono- and divalent anions > monovalent cations. The possible significance of this sequence, if any, is not clear.

Results finally indicated considerable difference in response of isotopes of similar charge. P^{32} (as $P^*O_4^{---}$) keenly reflected differences in concentration of external solution while response of S^{35} (as $S^*O_4^{--}$) was much more limited in this respect. Again, P^{32} loss was conditioned by particular ion species in the milieu while S^{35} showed no such response.

SUMMARY

Quantitative aspects of "short-term" P^{32} and S^{35} efflux from bean roots were studied. Techniques involved foliar application of isotope after which loss from roots to various single salt solutions was measured. A loss period of 50 minutes was adopted as standard procedure on the basis of preliminary time-course tests. In these tests, P^{32} and S^{35} loss occurred as an asymptote of time, and the period of initial rapid ("short-term") loss appeared completed after 50 minutes.

Large quantitative differences of logarithmic proportion existed between isotope applied to the foliage, found in the roots, and finally lost from roots. In very approximate terms, of every 1×10^6 atoms applied, only 1×10^4 atoms appeared in the root, and only 1×10^2 were lost from the root. Calculated as per cent of original root activity, loss values seldom exceeded the 1 per cent mark.

The point was emphasized that the quantitative relationships obtained as well as conclusions derived are specific to the particular techniques of the current experiment ("short-term" root efflux of foliar-applied isotopes to true solutions). Results differed when root introduction was practiced, and when loss occurred to a charged system,

Efflux of P^{32} was conditioned by species of ions in the ambient medium. Arranged in order of decreasing influence, ambient ions formed the sequence

$Mg^{++} > Cl^-$, NO_3^-, $SO_4^{--} > K^+$, NH_4^+ (divalent cation > mono- and divalent anions > monovalent cations). Root loss of S^{35}, on the other hand, was not altered by ambient ions.

Concentration of ions in the external medium also influenced P^{32} efflux. In all cases greatest P^{32} loss occurred at the high concentrations. No concentration influence was noted on S^{35} loss except for the magnesium solutions where greatest loss occured to the low concentrations.

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THE INFLUENCE OF RADIOACTIVE PHOSPHATE LEVEL ON THE ABSORPTION OF PHOSPHATE BY PLANTS AND ON THE DETERMINATION OF LABILE SOIL PHOSPHATE

by S. LARSEN and I. J. COOKE

Department of Soil Science, Levington Research Station, Levington, Ipswich, United Kingdom

INTRODUCTION

When radioactive phosphorus (P^{32}) is employed as an isotopic tracer in biological experiments it is important to ensure that the tracer has no significant effect on the results. This can be attempted by employing a "safe level" of P^{32} if such can be established.

Much of the work on the influence of P^{32} on plant growth in both solution and soil culture has been well reviewed by Steenberg [9] making it unnecessary to give a comprehensive review here. There is considerable divergence of opinion as to the influence of P^{32}, at levels required for the majority of tracer experiments, on plant yield, phosphorus uptake and the ratio of labelled (added) to unlabelled (native soil) phosphorus in the plant. Opinions vary from that of Russell *et al.*[7], who consider that the effects may readily invalidate experimental results, to that of Bould *et al.*[1], who conclude that overall effects are slight and in no way sufficient to preclude the use of P^{32} in plant-nutrition experiments with soil. Steenberg [9] has shown the marked effects which can occur at very high levels of P^{32}.

In pot experiments phosphate may be added to the soil in different forms and in different ways, *e.g.* as a solution applied to the soil surface or completely mixed with the soil, or as solid phosphates of varying solubility and particle size. The degree of incorporation in the soil will vary with the method of application and with the soil

properties and these factors will have a bearing on the possible effect of P^{32} on the absorption of labelled phosphate. The work of Russell *et al.*[8] and Mattingly [6] indicates that there is likely to be preferential phosphate uptake from regions of relatively low specific activity in the soil, leading to a reduced ratio of fertilizer-P to soil-P in the plant. This effect will inflate estimates of the labile soil phosphate determined as the *L*-value of Larsen [4] or the *A*-value of Fried and Dean [2], as Mattingly [6] has demonstrated. A case of particular interest arises if labelled phosphate is added as the phosphate form of an anion-exchange resin and uniformly mixed with the soil when, initially at least, the P^{32} will be distributed throughout the soil in discrete zones of high activity. If there is a reduced uptake of phosphate from these zones there will be an increased estimate of the labile phosphate in the soil. It is not possible to test this hypothesis directly as labile soil phosphate (*L*-values) cannot be determined without the use of P^{32} but the influence of increasing the level of P^{32} can be studied.

EXPERIMENTAL

Phosphate source. De Acidite FF (16–52 mesh, water regain 0.6–0.9 g per g, exchange capacity 2 milliequivs. per g) in the HPO_4^{--} form, labelled with the amount of $HP^{32}O_4^{--}$ required by the experimental treatment.

Soils. Four types were used. Their properties are shown in Table 1.

TABLE 1

Description of soils investigated			
Soil No.	Type	pH in water	Organic carbon, %
6	Sandy loam	6.5	2.2
8	Medium loam	7.1	2.4
9	Peat	5.9	16.2
18	Clay loam	6.8	2.1

Experimental design. Four P^{32}-levels (0; 0.05; 0.25 and 1.25 mc per g of phosphorus) replicated 3 times on each soil type.

Preparation of pots. Five-inch bituminized paper pots, each holding 1 kg of soil, were used except for soil 9 in which case $6\frac{1}{4}''$ pots were needed to contain 1 kg.

A quantity of the appropriately labelled resin containing 6 millimoles of phosphate was mixed thoroughly with 3 kg of soil and each of three pots was half filled with the mixture. Twenty-five millilitres of a solution containing 350 mg N and 195 mg K (as NH_4NO_3 and KNO_3) was added to each pot and

the filling of the pots with the soil—resin mixture was completed. The soil was compacted gently and 100 H1 ryegrass seeds were spread on the surface. The seeds were covered with a $\frac{1}{4}''$ layer of wet vermiculite which was kept moist to encourage germination by application of an occasional fine spray of water.

After preparation the pots were randomised on a bed of moist sand. The sand was kept moist during the growth of the crop and adequate water was supplied in this manner.

Brairding occurred after six days and cuts were taken 15, 31, and 73 days after sowing. The experiment was conducted rather late in the year and an attempt was made to obtain further cuts by transferring the pots to a heated glasshouse equipped with artificial lighting but the change of environment produced erratic growth and no further useful cuts were forthcoming.

After harvesting the crop samples were weighed, dried, and the dry matter weighed. The dry matter was then digested in a 2 : 2 : 1 v/v mixture of nitric, perchloric and sulphuric acids and the phosphorus content determined by a modification of the vanadomolybdate method described by Hanson [3]. The radioactivity of the sample was determined by counting the yellow solution obtained in the colorimetric phosphorus estimation in a type M6 liquid-counting tube, and the specific activity of the phosphorus calculated. The specific activity of the resin phosphate was determined in the same way.

The labile phosphate in the soils was computed from the equation

$$L = \left(\frac{C_f}{C_p} - 1 \right) X \qquad (1)$$

where C_f = specific activity of applied phosphate
C_p = specific activity of plant phosphate
X = quantity of applied phosphate (millimoles P per kg soil).

RESULTS

The principal results and their statistical analysis are shown in Tables 2 and 3. The fresh-weight, dry-matter, and phosphate-uptake figures are the sums of the values obtained for three cuts. L-values have been computed from the analysis of the third cut only, as the somewhat indeterminate influence of the seedborne phosphate is liable to cause error in L-values based on the first two cuts.

The figures show that increasing the level of P^{32} did not have a significant effect on the fresh weight, dry matter or phosphate uptake of the crop. The measured L-values increased significantly with increasing P^{32}-level on each of the four soil types investigated.

TABLE 2

Effect of P^{32}-level on yield of fresh and dry weight of ryegrass and on phosphorus uptake

Soil or P^{32}-level	Totals of three cuts		
	Fresh weight g per pot	Dry matter g per pot	Phosphorus in dry matter mg per pot
Soil: 6	15.75	1.73	11.32
8	19.29	2.10	13.01
9	18.24	1.92	11.30
18	16.94	1.79	12.96
P^{32}-level: Nil	17.38	1.90	11.97
0.05 mc per g P	18.17	1.95	13.08
0.25 mc per g P	16.76	1.74	11.22
1.25 mc per g P	17.92	1.96	12.32
L.S.D. ($P = 0.05$)	1.87	0.20	1.46
L.S.D. ($P = 0.01$)	2.52	0.27	1.97
Coefficient of variation (%)	12.73	12.51	14.38

TABLE 3

Effect of P^{32}-level on the L-value (millimoles P per kg of soil) of four soils (cut 3)

Soil: 6	11.25
8	7.27
9	3.66
18	12.03
L.S.D. ($P = 0.05$)	0.90
L.S.D. ($P = 0.01$)	1.23
P^{32}-level: 0.05 mc per gP	7.18
0.25 mc per gP	8.77
1.25 mc per gP	9.72
L.S.D. ($P = 0.05$)	0.78
L.S.D. ($P = 0.01$)	1.07
Coefficient of variation (%)	12.48

DISCUSSION

The findings are in agreement with the hypothesis developed from the results of related work in the published literature. The effect of P^{32} on L-value can only have been brought about by an impeded absorption of labelled phosphate which would reduce the specific activity of the plant phosphorus, *i.e.* C_p in Equation (1).

There is no indication of a threshold (safe) level of P^{32} below which

it has no influence on the result. If one exists it is presumably less than the lowest level used, *viz* 0.05 mc per g P (equivalent to about 3 μc per pot) at the time of sowing, which is a rather low level for convenient experimental use. This is compatible with the findings of Mackie *et al.*[5] who were unable to demonstrate a threshold value for P^{32} when studying histological damage caused by this isotope to barley plants.

Although a significant effect of P^{32} on *L*-value determination was observed it was consistent on the different soils studied and did not invalidate their comparison with respect to *L*-value.

SUMMARY

Previous work has suggested that the presence of P^{32} in fertilizers inhibits the uptake of the applied phosphate from the soil by plants, and also that if the applied phosphate is not incorporated uniformly in the soil there will be preferential uptake from regions of low specific activity. This made it desirable to determine the effect of P^{32}-level on phosphate uptake and the determination of *L*-values in pot experiments in which the labelled phosphate source is added as discrete particles of the phosphate form of an anion-exchange resin.

Increasing the level of P^{32} from 0.05 to 1.25 mc per gram of phosphorus in the added phosphate did not have a significant effect on the fresh weight, dry weight or total phosphorus uptake of the ryegrass crop. The measured *L*-value showed a significant increase, about 15 per cent for a five-fold increase in P^{32} level, on each of the four soil types used, as would be expected if P^{32} depressed the uptake of labelled fertilizer phosphate.

Although a significant effect of P^{32} was observed this does not invalidate a comparison of soils with respect to *L*-value.

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STUDIES ON NITROGEN-FIXING BACTERIA OF THE GENUS *BEIJERINCKIA*

I. GEOGRAPHICAL AND ECOLOGICAL DISTRIBUTION IN SOILS

by J. H. BECKING

Laboratory of Microbiology, Agricultural University,
Wageningen, The Netherlands

INTRODUCTION

The genus *Beijerinckia* comprises the specific aerobic nitrogen-fixing bacteria also known as *Azotobacter indicum* Starkey and De [34]. In 1950 Derx [5] – at that time working at the Botanical Gardens at Bogor, Java (Indonesia) – found this microbe in some Bogor soils and proposed the new generic name of *Beijerinckia* for it in view of differences in morphology and cultural characteristics that distinguish it from the other *Azotobacter* species. In addition to the type species *Beijerinckia indica* (Starkey and De, Derx *comb. nov.*), Derx [6] has since added to this genus a new species, *viz B. mobile* and a variant, *B. indica* var. *alba*.

In contrast to the nitrogen-fixing bacteria of the genus *Azotobacter*, there is very little information available on the distribution of representatives of the genus *Beijerinckia* in soils. Reports on isolations of bacteria of the latter genus are seldom encountered in the literature. After its original isolation by Starkey and De [34] most of the new isolations seem to have been accidental, with the exception of the work of Derx [5 6], Tchan [38], and Döbereiner and De Castro [7]. Derx [6] isolated many Beijerinckia strains from the Bogor soils and estimated the number of Beijerinckia cells in a wet-rice (sawah) soil profile, making a comparison with Azotobacter numbers in the same soil. Tchan [38] studied the distribution of Beijerinckia in soils of northern Australia, while Döbereiner

and De Castro [7] made a survey of the occurrence of Beijerinckia in some soils of the state of Rio de Janeiro in Brazil.

Summarizing the available data, Beijerinckia has been reported from the following localities:

India (Dacca) original isolation by Starkey and De [34], Burma (Insein) [34], Malaya (probably isolated by Altson [1]), Indonesia (Bogor, W. Java) [5 17], Japan (Sendai) [35 36], North Australia (north of latitude 17–18° S.) [38], Ivory Coast (Abidjan) [15]. Madagascar (Tananarive) [8], Tanganyika [19 20], Suriname (Paramaribo) [17] and Brazil (state of Rio de Janeiro) [7]. In addition, Ruinen [30] reported the occurrence of Beijerinckia on the surface of leaves ("phyllosphere") of tropical plants in Indonesia, while recently Roy [29] described a new acid-tolerant *Azotobacter* species (*A. acidum*) from a red earth of Madras (Pattambi) in India, which is probably a *Beijerinckia* species.

The conditions affecting the occurrence of Beijerinckia in soils are apparently different from those known for Azotobacter. Most isolations of Beijerinckia have been made from acid soils in which Azotobacter was entirely absent. Although some exceptional Azotobacter strains (*A. beijerinckii* var. *acido-tolerans* [37] and *A. macrocytogenes* [14] have been isolated which are able to grow and to fix nitrogen at pH values of 4.5 to 5.0, Beijerinckia is obviously far more acid tolerant. Moreover, nearly all Beijerinckia-positive soils were found to occur in tropical regions. A limited geographical distribution seemed to be unique for these soil microbes.

The present study forms a geographical and ecological survey of the distribution of Beijerinckia in soil samples of very different origin. For purpose of comparison, the presence of Azotobacter in the same soil samples was determined. Some factors which presumably influenced the distribution of Beijerinckia were tested in some detail.

MATERIAL AND METHODS

About 400 soil samples of different origin were examined for the presence of *Beijerinckia* spp. and *Azotobacter* spp. A complete list of the soil samples tested and some details with regard to soil type, soil pH, and cover are given in Table 1. The soil pH was measured by a glass electrode in the soil paste obtained by stirring soil with distilled water.

Beijerinckia spp. were determined in the soils by means of a selective, acid, nitrogen-free enrichment medium of the following composition: glucose 20.0 g, KH_2PO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, and distilled water

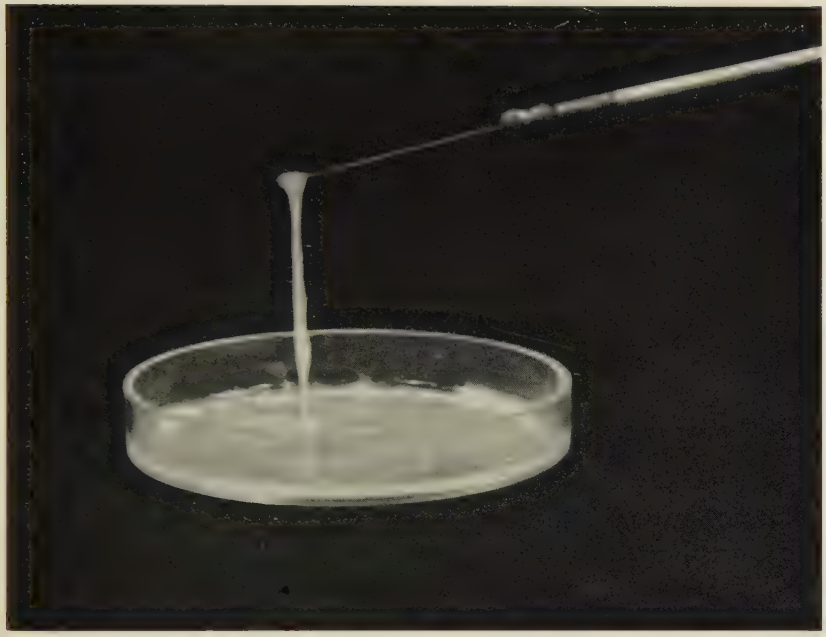

Plate 1. Enrichment culture of Beijerinckia in a petri dish containing a thin liquid layer (2 to 3 mm) of medium inoculated with soil, showing the very viscous consistency of the medium after three weeks.

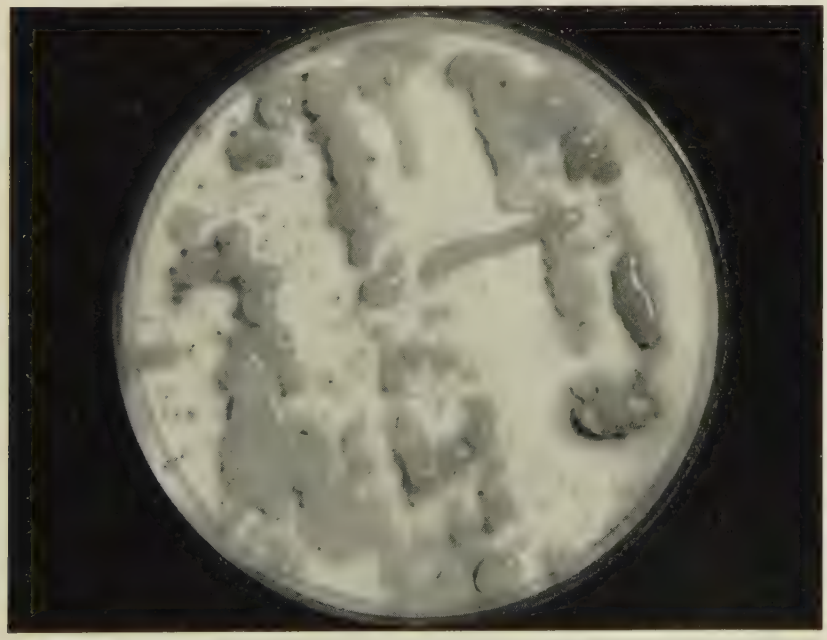

Plate 2. Culture of *Beijerinckia* species obtained by plating out an enrichment culture. The plate shows the very slimy, highly raised, glistening Beijerinckia colonies and some fungus growth. The slime produced by Beijerinckia is very tenacious and elastic, which makes it very difficult to remove from the agar surface.

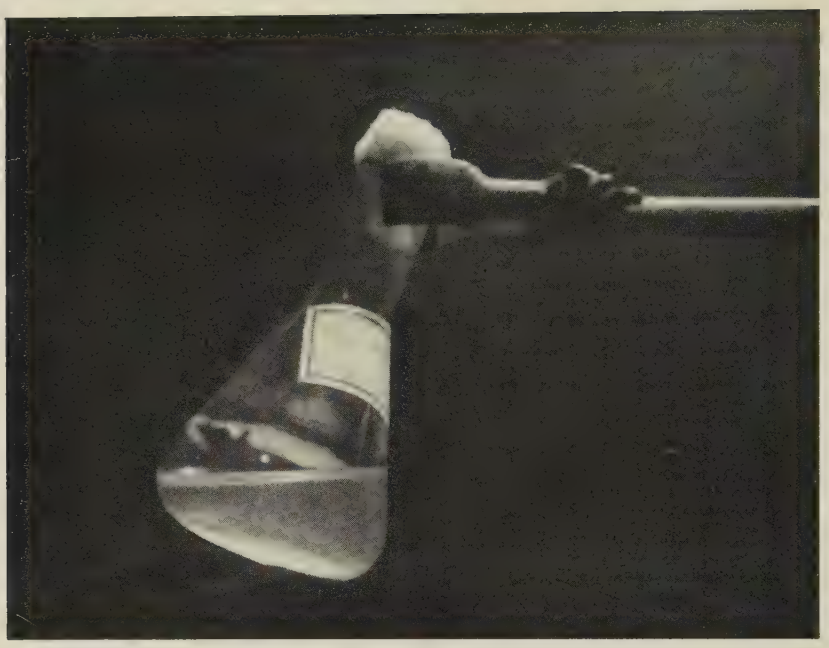

Plate 3. Growth of Beijerinckia in an erlenmeyer flask. Although the entire medium has become homogeneously viscous through the formation of a semi-transparent mucus, the latter adheres somewhat more to the wall of the flask, forming a continuous ring near the surface. But Beijerinckia does not show the distinct surface pellicle characteristic for Azotobacter.

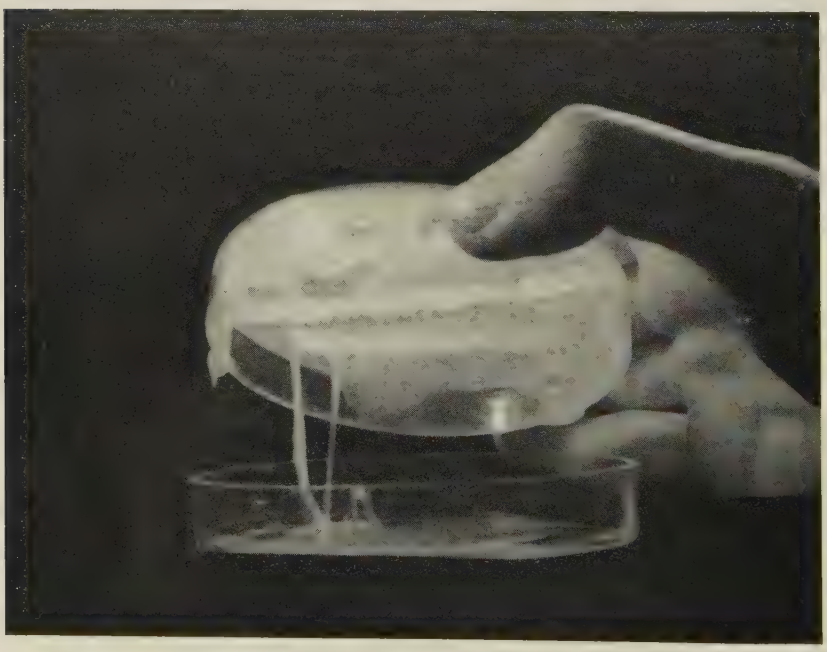

Plate 4. Pure culture of Beijerinckia cultivated in a reversed petri dish. The development of slime is so luxuriant that it drips into the reversed cover.

1 litre (pH 5.0). The medium was poured in very thin layers (2 to 3 mm) into petri dishes. Approximately 0.5 g of soil was used as inoculum per dish. All soils were tested in 10 replicates. After incubation for two or more weeks at 30°C the cultures were examined microscopically. As a rule, the presence of Beijerinckia in the medium could be established simply by the appearance of the medium. In an advanced stage of development the entire medium changed into a viscous slimy mass which could be drawn out into long threads (see Plate 1).

A positive result on the basis of microscopic examination was always checked by plating the enrichment culture on acid, nitrogen-free agar plates containing: 2% agar, 2% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $FeCl_3.6H_2O$, and 0.002% $Na_2MoO_4.2H_2O$ (pH 5.0). On these plates Beijerinckia developed characteristic, highly raised, glistening colonies of a very elastic and tough slime (see Plates 2 and 4). Only some of the Beijerinckia strains (*Beijerinckia mobile* [6]) produced flatter, Azotobacter-like colonies, but with a different, more reddish brown colour.

The presence of *Azotobacter* spp. in the soil samples was determined with a medium in which the acid KH_2PO_4 was replaced by the same amount of alkaline K_2HPO_4, and 2% $CaCO_3$ was added because Azotobacter requires calcium for development. The enrichment cultures were plated on alkaline (pH 7.5), nitrogen-free, glucose agar plates.

Since most of the soils were derived from foreign regions, there was in most cases a delay of a few weeks to several months between soil sampling and microbiological examination. Such a delay, however, did not appreciably affects the results, since both Beijerinckia and Azotobacter were found to be very persistent in soils in which they occur naturally. Both species could still be isolated from soils stored for more than 5 years under air-dry conditions at room temperature in the temperate region.

RESULTS

1 DISTRIBUTION OF BEIJERINCKIA AND AZOTOBACTER IN SOILS OF DIFFERENT ORIGIN

The present survey covers the examination of 392 soil samples of different origin - 155 European, 101 African, 51 Asiatic, 30 Australian, 3 North American, and 52 South American. Of these soils, 244 were derived from temperate and 148 from tropical regions. Tables 1, 2, and 3 show the results obtained.

Of the 155 soils of European origin only two (1.3 per cent) contained Beijerinckia. Both Beijerinckia-positive soils were collected in two neighbouring localities: Medvea and Kostrena near Rijeka (Fiume) in Yugoslavia close to the Adriatic coast.

TABLE 1

Distribution of Beijerinckia (B) and Azotobacter (A) in soils of different origin (n = no. of soils examined)

Locality	Soil type	pH	Cover	n	B	A
EUROPE						
Netherlands						
Provinces Gelderland (Wageningen), Drenthe and Brabant (Esbeek)	Sandy soil	4.5–4.9	Arable land	20	0	0
	Sandy soil	3.4–5.7	Forest plantations	20	0	0
	Sandy soil, with podzol soil profile	3.2–4.5	Heather *etc.*	10	0	0
	Sandy soil, heavily dressed and manured	7.0–7.5	Garden	3	0	3
Prov. Groningen and Drenthe	Lowmoor and highmoor peaty soils including ironstone-containing Mo-deficient lowmoor soil (cf Mulder [24])	4.8–5.6	Incultivated, arable land and pasture	15	0	0
Prov. Gelderland (Wageningen)	River clay, Rhine border	6.9–7.2	Arable land and grassland	12	0	12
Prov. Groningen	Acid marine clay	4.6–5.9	Arable land and grassland	10	0	0
Prov. Zuid-Holland	Alkaline marine clay	6.9–7.6	Arable land and grassland	10	0	10
Prov. Zuid-Holland, Gelderland	Ditch mud	7.2–7.5		4	0	2
	Greenhouse soil	7.0–7.9	Tropical plants and rice	4	0	4
Germany						
Prov. Bayern (Chiemsee)	Black peat, highmoor	3.8–5.5	Uncultivated	2	0	0
Switzerland						
Lausanne, Lac Leman	Clay soil	7.7	Arable land	3	0	3
France						
Rhône delta	Heavy gray clay, rice soil	7.5–7.7	Rice	4	0	4
Mediterranean region (Cannes, Monaco etc.)	"Terra rossa" soils, weathering product of lime stone	7.3–7.7	Uncultivated, and arable	15	0	15
	The same sample containing much humus	3.4	Garden	1	0	0
Italy						
Southern region (Foggia, Bari, Taranto)	"Terra rossa" soil	7.3–8.0	Grape	12	0	12
Yugoslavia						
Skopje, Dubrovnik, Isl. Vis and Hvar, Medvea and Kostrena near Rijeka	"Terra rossa" soil	6.8–8.0	Arable land and uncultivated	10	2	10
AFRICA						
Algeria						
El Biar	"Terra rossa" soil	7.7	Arable land			

Tamera	"Terra rossa" soil, more acid sub-soils	5.1–7.3	Arable land and exposed sites	3	0	
Egypt						
Nile delta (Abou El Matami, El Khazan Farm)	Heavy clay, Nile deposit	7.6–7.9	Arable land	3	0	3
Alexandria, Alexander Farm, Univ. of Alexandria	Fine sand	8.0	Arable land	1	0	1
Gambia						
Yundum Farm, Exp. plots	Fine sand, unfertilized (cf Webb [39]) deficient in N, P, and Mo.	5.2	Various crops	1	0	0
Sierra Leone						
Freetown area (Kissy district)	Lateritic loam, true "latosol"	5.9	Local grassland	1	1	0
	Lateritic loam, true "latosol"	5.3	Secundary forest	1	1	0
	Lateritic loam, true "latosol"	6.4–6.8	Arable land: cassava and sweet potatoes	2	2	1
	Lateritic loam, true "latosol"	5.0	Exposed site	1	1	0
Nigeria						
Ndop Plain near Bamali (alt. 1300 m)	Heavy clay, swamp soil, dry in dry season	5.7	Elephant grass	1	0	0
Ibadan-Iee road (W. region) Production Development Board Farm	Fine loam, rice soil	6.5	Rice	1	1	1
Ondo-Ife road	Litter-rich forest soil	4.4	Tall trees	1	0	0
Cameroons						
Bamenda						
Binka-Nkambe road (alt. 1800 m)	Ferruginous earth, more or less lateritic soil	5.7	Grassland and bracken	1	1	0
Kumbo-Ndu road (alt. 1900 m)	Ferruginous earth, volcanic bedrock	5.3	Grassland, now broken up for cultivation	1	1	0
Ethiopia						
E. reg.: Harrar, Jigjigga, Lake Aramaya, Dire Dawa (alt. 1600–2300 m)	Red lateritic sand, rocky region	6.0–6.4	Tropical thornland	3	3	1
	Red lateritic sand, probably dressed	7.6–7.7	Cultivated, cotton plantation	2	0	2
C. reg.: Addis Abeba, Entoto-hills, Gulele, Kafarza (alt. 2450–2800 m)	Red lateritic loam	4.9–6.3	Local grassland, exposed sites	11	6	1
S. reg.: Colaris, Avela Wando (alt. 1850–1920 m)	Red lateritic loam (rhyolitic origin)	6.1–6.6	Coffee plantations	2	1	0
Irgalem, Dilla (alt. 1650–1850 m)	Red lateritic loam	5.0–5.3	Uncultivated	3	3	0
W. reg.: Baco, Ambo, Lechemti (alt. 1700–2300 m)	Red lateritic loam (olivine-basalt origin)	5.3–5.9	Local grassland and *Eucalyptus* trees	5	3	0
N. reg.: Debra Sina	Red lateritic loam	6.2	Uncultivated	1	0	0
Kenya						
Kikuyu (alt. 2100 m)	Lateritic loam, darker and more fertile	6.5	Rank grass, following removal of wattle	1	0	0

TABLE 1 (continued)

Distribution of Beijerinckia (B) and Azotobacter (A) in soils of different origin (n = no. of soils examined)						
Locality	Soil type	pH	Cover	n	B	A
Ol Joro Orok, N.E. of Nakuru (1800–2400 m)	Grey soil, seasonnally-wet	5.0	Local grassland	1	0	0
	Dark loam, containing much humus	6.7	Wheat land, soon after harvesting	1	0	1
	Reddish-brown lateritic soil containing pisolitic ironstone	5.5	Old arable land	1	1	0
Uganda						
Kampala, Kawanda Res. Station (alt. 1200 m)	Red lateritic loam (Soil structure plot 1)	5.0	Various crops	1	1	0
Mt. Elgon (alt. 1700 m)	Forest soil (conglomeritic lava origin)	6.2	Natural forest	1	0	0
Mabira forest, Tinja (alt. 1200 m)	Forest soil (amphibolitic origin)	6.5	Natural forest	1	0	0
Kigezi district (S.W. region), Gorilla sanctuary (alt. 1800 m)	Forest soil, extremely acid yellow laterite	3.0	Natural forest	1	0	0
Ankole district, Kanahunga	Black earth, rain-forest edge	4.3	Bracken, *Dissotis* and *Philipia*	1	0	0
Tanganyika						
Ukiriguru, Lake district	Lateritic loam, "Immature red skeletal soil" (cf. Morrison *et al.* [23])	5.6	Cultivated	1	1	0
	Black earth, Illuvial soil	7.3	Cultivated	1	0	1
	Coarse red sand, "Upper coluvial soil" [23]	6.9	Uncultivated	1	1	0
	Fine sand, "Lower coluvial soil" [23]	7.9	Cultivated	1	0	1
	Black heavy silt, Alluvial soil, "Mbuga" [23]	7.2	Cultivated	1	1	1
Congo						
Brazzaville	Garden soil	7.4	Garden	1	1	1
South Africa						
Transvaal, Swaziland, Natal	Mostly lateritic loams (cf Van der Merwe [21], for more details see Ref. [2])	4.2–7.7	Forest plantations	21	12	3
Cape Province	Diverse soil types, see Becking [2]	3.6–7.5	Mostly forest plantations	19	3	2
ASIA						
India						
Pusa, near New Delhi	Heavy clay, irrigated by Ganges	7.8–8.1	Rice and corn	2	0	2
New Delhi, Hardwar	Sandy soil	8.2	Sugar cane	1	0	1
Koelpura, N. of Hardwar	Forest soil (on Siwalik conglomerates and sand rocks)	5.7	Rape	1	0	0
		6.5	*Shorea robusta* plantation	1	1	0

Mohand, S.W. Dehra Dun	Sandy soil	7.4	Taungya culture			
Dehra Dun	Brown loam	6.6–8.0	Forest plantation and peanut cultivation	2	0	1
		Mats.				
Coonoor, Ootacamund, E. Nilgeris	Ferruginous loam	6.8	Irrigated *Areca* palm garden	1	0	1
Calicut, W. of Nilambur, Madras	Lateritic loam (gneiss origin)	6.5	*Tectona grandis* forest, 109 years old	1	1	0
China						
Hongkong, Lamma Isl.	Sandy loam (from Tai Mo Shan porphyry cf Davis [4])	7.4	Cultivated	1	1	1
	The same	6.1	Uncultivated	1	1	0
Hongkong, Ping Chau Isl.	Marine sediment (Tolo Channel Sediment [4])	7.7–7.9	Cultivated and uncultivated	2	0	2
Hongkong, Chu Lu Kok Isl.	Sandy loam (granitic origin [4])	5.6–5.7	Cultivated and uncultivated	2	2	0
Kwantung Prov., Hainan Isl.	Red lateritic loam (granitic origin)	5.3	*Hevea brasiliensis* with *Paspalum conjugatum* as cover grass	1	1	0
Lai-Chou Peninsula	Red lateritic loam (basaltic origin)	5.6	Irrigated rice	1	1	0
		5.1	Secondary forest	1	1	0
Japan						
Shiono, near Karuizawa (alt. 1000–1100 m)	Fine loam, forest soil	5.5–6.0	*Larix japonica* plantations	2	0	0
Nikko National Park, plateau above Chuzenji Lake (alt. 1300–1500 m)	Sandy loam, volcanic-ash soil	5.3–5.8	Virgin *Larix* forest	2	1	0
Nikko Botanical Garden (alt. 650 m)	Sandy loam, volcanic-ash soil	5.0	Garden	1	1	0
Okorogawa, S. of Nikko (alt. 430 m)	Heavy reddish-yellow loam, volcanic-ash soil	5.7–6.5	*Cryptomeria* plantations, 80 years old	2	2	0
Tokyo (alt. 10 m)	Sandy soil	4.8	Botanical gardens	1	1	0
Hawaii Islands						
Oahu, Waimanalo	Red lateritic loams, "Latosol" (cf Sherman [18 32])	4.8	Iron-wood trees	1	0	0
Oahu, Poamoho	The same, "Low humic latosol" [18 32]	5.6	General crops	1	1	0
Oahu, Kaneohe	The same, "Humic latosol" [18 32]	5.2	Pasture	1	1	0
Oahu, Kunia	The same, "Humic ferruginous latosol" [18 32]	4.4	Pineapple	1	1	0
Indonesia						
Bogor, W. Java	Lateritic loam (andesitic-tuff origin [22])	5.2–6.5	Irrigated rice, garden and forest	3	3	1
Tjibinong, Exp. Gardens, N.E. of Bogor	Very senile lateritic loam (andesitic-tuff origin [22])	4.8	Arable land	1	1	0
Tjibodas, Gng. Pangarango, W. Java	Forest soil	5.6	Virgin forest	1	1	0
Tandjung Priok, W. Java	Clay (river and marine origin)	7.7–8.0	Uncultivated and cultivated	2	0	2
Ngawi, C. Java	Heavy black clay, margalite soil (cf Mohr [22])	6.1		1	0	1
Surabaya, E. Java	Marine sediment and clay	7.2–9.0	Pasture and garden	2	0	2

TABLE 1 (continued)

Distribution of Beijerinckia (B) and Azotobacter (A) in soils of different origin (n = no. of soils examined)						
Locality	Soil type	pH	Cover	n	B	A
Belawan, N. Sumatra	Marine clay	7.7	Arable land	1	0	1
New Guinea						
Hollandia, Reinauwen (Camp Walker, Dok 5)	Red lateritic loam (serpentine and schists origin)	5.4–5.9	Uncultivated and cultivated	4	3	0
Reinauwen (swamp)	Organic litter-rich swamp soil	6.1	Uncultivated	1	1	0
Cyclope Mountains (alt. 1200–1630 m)	Senile lateritic loam	4.4	Virgin forest	1	0	0
Kebar valley, Nettoti-ridge (alt. 600–1000 m)	Very senile yellow loam	4.0–4.4	Virgin (o.a. *Auracaria*) forest	3	0	0
AUSTRALIA						
South Australia						
Mt. Graham	Gray loam (volcanic soil), forest soils litter-rich	5.3–5.5	Pasture, cultivated and forest plantations	3	0	0
Mt. Burr	Red sandy loam, containing humus, "terra rossa" (Mt. Muir sand)	5.3–5.5	Local pasture and forest plantation	3	0	0
3 miles E. of Millicent	Sandy soil (Riddoch sand)	4.9–5.8	The same	3	0	0
1 mile E. of Millicent	Heavy clay, margalitic (rendzina)	7.7	Pasture and arable land	2	0	2
Tantanoola, S. of Millicent	Red loam and sandy loam (flinty sand)	4.9–6.4	Local pasture, cultiv. and forest	5	0	1
Victoria						
1.5 miles W. of Dunkeld	Red sandy loam	5.1–5.3	Pasture and *Eucalyptus* plantations	2	0	0
4 miles W. of Camperdown	Loam (volcanic soil)	5.1–5.5	Arable, pasture and uncultivated	3	0	0
10–25 miles W. of Bendigo	Red loam	6.2–6.3	The same	3	0	2
4 miles E. of Stanhope	Red loam	5.6–5.8	Irrigated pasture and arable land	3	0	0
New South Wales						
23 miles E. of Deniliquin	Red loam	5.6–6.1	Irrigated clover pastures	3	0	0
NORTH AMERICA						
United States						
Florida, Miami	Peaty soil, „Muck"	7.6	Uncultivated	1	0	1
	Grey silt containing shell fragments, Marl soil	7.9	Uncultivated	1	0	1
	Coral rock soil	8.3	Uncultivated	1	0	1

SOUTH AMERICA						
Trinidad Isl. (Antilles)						
Centeno	Compact clay	5.5	Sugar cane	1	0	0
Caroni	Compact clay	7.8	Sugar cane	1	0	1
San Rafael	Lateritic loam	4.8	Cocoa	1	1	0
Las Lomas	Yellow clay	4.4	Citrus	1	0	0
Venezuela						
Merida, Cord. de Merida (alt. 1170 m)	Red loam, forest soil	3.9	Virgin forest (a.o. *Podocarpus rospigliossii*)	1	0	0
Suriname						
Coastal region, Paramaribo	Compact clay	5.1	Rice	1	1	0
	Yellow lateritic fine sand	5.6	Cultivated	1	1	0
	Yellow podzolic fine sand soil, intermittantly under action of ground water	4.5	Forest	1	0	0
Central region, Voltzberg (Coppename river)	Red lateritic loam (biotite granite origin)	4.0	Tropical rain-forest	1	0	0
	The same	4.2 4.8	Savannah and savannah forest	4	4	0
Bolivia						
Santa Cruz (alt. 500 m) Est. Exp. Agric. Los Llanos	Clayey loam	5.4–6.2	Rice, sugar cane, corn, local grassland and natural forest	5	2	0
Riberalta, Beni (alt. 100–200 m) Est. Exp. Y Vecindades	Red sandy loam, lateritic	4.8 6.2	Rice, sugar cane, cassava, natural forest and introduced grasses	5	3	0
Reyes, Beni (alt. 300 m)	Yellow brown loam	5.3 7.0	The same	5	1	3
Trinidad, Beni (alt. 200–500 m)	Heavy compact clay	7.1–7.6	Rice, corn, Yuca and natural forest	4	0	4
Brazil						
Northern (Amazon) region:						
Pará: Belém, Capanema, Belterra.	Red lateritic loams, partly very senile	4.2–6.2	Cultivated, uncultivated	10	3	0
Amapá: Amapá, Bom Jesus.	Very senile lateritic loam	4.2–4.7	Cultivated, uncultivated, virgin forest	4	0	0
Amazonas: Manaus, Paredão.	The same	3.9–4.5	The same	2	1	0
Southern region:						
Rio de Janeiro, "Corcovado" rock	Secundary forest soil	5.8	Trees	1	0	0
Rio de Janeiro, Tijuca Lake	Alluvial soil, rich in organic matter	6.2	Grass and reeds	1	0	1
São Paulo	Garden soil	6.9	Garden	1	1	1
Ponta Grossa	Red sandy loam	5.1	Formerly local grassland, now dry rice cultivation	1	1	0

TABLE 2

The number of soils of different regions tested and the number of Beijerinckia- and Azotobacter-positive soils in each region

Region	Number of soils		
	Examined	Beijerinckia positive	Azotobacter positive
Europe			
Northern (The Netherlands, Germany, *etc.*)	110	0	31
Southern (Mediterranean region)	45	2	44
	155	2	75
Africa			
Tunis, Algeria	4	0	2
Egypt	4	0	4
Gambia	1	0	0
Sierra Leone	5	5	1
Nigeria	3	1	1
Cameroons	2	2	0
Ethiopia	27	16	4
Kenya	4	1	1
Uganda	5	1	0
Tanganyika	5	3	3
Congo	1	1	1
South Africa	40	15	5
	101	45	22
Asia			
Japan	8	5	0
India	10	2	6
China	9	8	3
Hawaii Islands	4	3	0
Indonesia	11	5	7
New Guinea	9	5	0
	51	28	16
Australia			
Southern (South Australia, Victoria New South Wales)	30	0	5
North America			
Florida	3	0	3
South America			
Trinidad	4	1	1
(Antilles)		0	0
Venezuela	1	6	0
Suriname	8		
Bolivia	19	6	7
Brazil:			
Northern	16	4	0
Southern	4	2	2
	52	19	10

TABLE 3

The number of soils of the different regions tested and the percentage of soils containing either *Beijerinckia* (B), *Azotobacter* (A), both species, or neither

Region	Number of soils tested	Percentage of soils containing			
		B	A	B + A	no B or A
Europe					
Northern (The Netherlands, Germany)	110	0	28	0	72
Southern (Mediterranean region)	45	4	98	4	2
Total	155	1	48	1	52
Africa					
Northern (Tunis, Algeria, Egypt)	8	0	75	0	25
Tropical	53	57	21	11	34
Southern (South Africa)	40	38	13	5	55
Total	101	45	22	8	42
Asia					
Tropical (India, China, Hawaii Isl., Indonesia, New Guinea)	43	53	37	5	16
Temperate (Japan)	8	63	0	0	37
Australia					
Southern (South Australia, Victoria, New South Wales)	30	0	17	0	83
North America					
Florida	3	0	100	0	0
South America					
Tropical (Trinidad Isl., Suriname, Venezuela, Bolivia, Brazil)	52	37	19	4	48

It should be stressed that in these Beijerinckia-positive soils any contamination from other sources was excluded. The present observation seems to be the first record of the occurrence of Beijerinckia in Europe; it shows a distribution as far north as latitude 45° N. Twenty-eight per cent of the north European soils yielded Azotobacter, but the percentage of south European soils containing this

micro-organism was far higher (98 per cent). This difference is due to the predominant presence in this region of the alkaline "terra rossa" soils, which are a weathering product of limestone.

Of the 101 African soils tested, which represent this continent from north to south, Beijerinckia was absent in the soils of the northern part (Tunis, Algeria, and Egypt), but 75 per cent of these soils contained Azotobacter abundantly. Of the 53 soils tested from the central, tropical part of this continent, 57 per cent yielded Beijerinckia and only 21 per cent Azotobacter. However, still further south the number of Beijerinckia-positive soils decreased again: of the 40 South-African soils tested, 38 per cent contained Beijerinckia and 13 per cent Azotobacter. The distribution of Beijerinckia in South Africa was largely confined to the lateritic soils of the so-called "Mist-belt" in northern Transvaal (Lat. 23° to 25° S.). This microbe was, however, also found, although far more sporadically, in some other soil types as far south as Cape Town (Lat. 34° S.). A more detailled account of the occurrence of Beijerinckia in South African soils has been given in a preceding paper [2].

Of the Asiatic region, 51 soil samples, mostly derived from the tropical region, were examined. Of these soils, 53 per cent gave Beijerinckia and 37 per cent Azotobacter growth. Only 8 soil samples were obtained from Japan, which has a temperate climate. Very remarkable was the rather common distribution of Beijerinckia in the Japanese soils, since of the 8 samples collected at Honshu Island, 5 were Beijerinckia positive. Although these localities were at rather high latitudes (Lat. 35° to 37° N.), some of the positive samples from Nikko National Park (alt. 1300 to 1500 m) were covered with melting snow at time of collecting (March).

Beijerinckia was isolated from a soil of Koelpura (Lat. 30° N.) at the base of the Himalaya Mountains in northern India, a locality with a very prolonged cold season but where the summer months are almost tropical in their extreme heat. It was also found in some mountain forest soils at high altitudes (alt. 1500 to 3000 m) in Java, Indonesia.

From the South American continent, 52 soil samples were examined from localities extending from latitude 10° N. to 23.5° S. Of these soils 37 per cent contained Beijerinckia and only 19 per cent Azotobacter.

TABLE 4

The distribution of Azotobacter and Beijerinckia in non-tropical soils

Region	Number of soils tested	Beijerinkia positive	Azotobacter positive
Europe	155	2	75
Northern Africa (Tunis, Algeria, Egypt)	8	0	6
South Africa	40	15	5
Asia (Japan)	8	5	0
South Australia (South Australia, Victoria, New South Wales)	30	0	5
North America (Florida)	3	0	3
Total	244	22	94
Percentage	100	9.0	38.5

TABLE 5

Distribution of Beijerinckia and Azotobacter in tropical soils

Region	Number of soils tested	Beijerinckia positive	Azotobacter positive
Africa	53	30	11
Asia	43	22	16
South America	52	19	10
Total	148	71	37
Percentage	100	48.0	25.0

In four soil samples from Florida which has a sub-tropical climate, Beijerinckia was entirely absent, but these alkaline soils gave profuse development of Azotobacter.

Of the 30 Australian soils, derived from the southern part of this continent (Lat. 36° to 38° S.) which has a temperate climate, none contained Beijerinckia and 17 per cent Azotobacter.

Tables 4 and 5 show that for the temperate region only 9 per cent of the samples contained Beijerinckia and 38.5 per cent Azotobacter, whereas the tropical soil samples contained 48 per cent Beijerinckia and 25 per cent Azotobacter.

2 EFFECT OF TEMPERATURE

A limitation of Beijerinckia distribution to the tropics might be caused by a preference for the higher temperatures of this region. This suggestion conflicts, however, with the observations

mentioned in the soil survey showing the occurrence of Beijerinckia in some soils of Japan, northern India, and South Africa, and its common distribution in some tropical mountain soils in Asia (Java) and Africa (Ethiopia, Kenya, *etc.*) at very high altitudes.

In view of this discrepancy, it was interesting to test the effect of temperature on the growth and survival of Beijerinckia in some detail. Experiments were performed with some Beijerinckia strains freshly isolated from soils collected at different latitudes and altitudes. For comparison, some Azotobacter strains of both temperate and tropical origin were included in the experiments.

(a) *Growth experiments*

The Beijerinckia strains were grown in a nitrogen-free glucose medium containing: glucose 2%, K_2HPO_4 0.08%, KH_2PO_4 0.02%, $MgSO_4.7H_2O$ 0.05%, $FeCl_3.6H_2O$ 0.005%, $Na_2MoO_4.2H_2O$ 0.0005%, and distilled water 1 litre, (pH 6.9). The Azotobacter strains were grown in a medium identical, except that the phosphate was replaced by K_2HPO_4 0.1% and $CaCO_3$

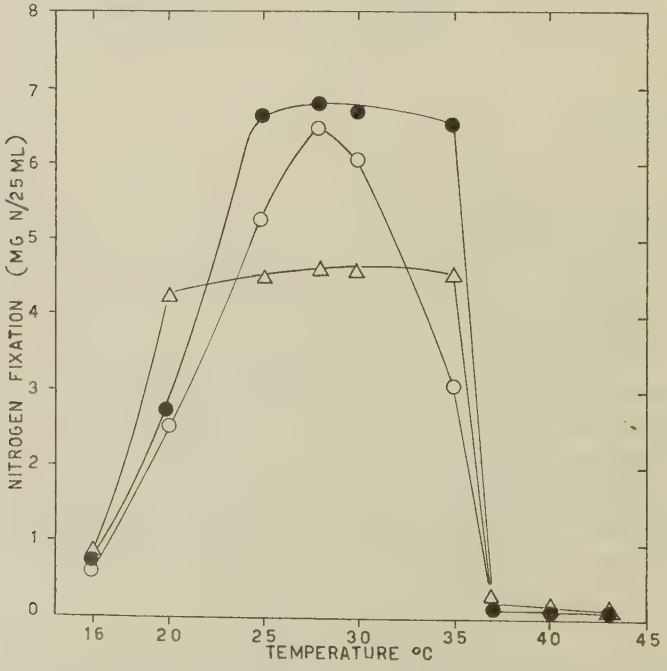

Fig. 1. Nitrogen fixation in relation to temperature in three *Beijerinckia indica* strains, *viz* ● str. Hawaii 3/1, ○ str. Bogor Java 8, △ str. Ehtiopia Λ 1/1.

0.04% was added (pH 7.2). The medium was pipetted in 25-ml aliquots into 100-ml erlenmeyer flasks and after inoculation all strains were incubated at a range of temperatures of 16°, 20°, 25°, 28°, 30°, 37°, 40°, and 45°C. Nitrogen gains were determined by the conventional micro-Kjeldahl procedure following the modification of Yuen and Pollard [41]. The Beijerinckia cultures were harvested after incubation for 35 days and Azotobacter after 14 days. Tests with each strain were performed in five replicates; the average values are given in the Figures.

The following *Beijerinckia indica* strains were selected: (1) str. Hawaii 3/1 isolated from a "Humic Latosol" [18 32] of Kaneohe (alt. 60 m), Oahu, Hawaii Islands, (2) str. Bogor 8 from a senile laterite soil [22] of Bogor (alt. 250 m), W. Java, Indonesia, and (3) str. Ethiopia A 1/1 from a red earth of Harrar-Jigjigga (alt. 1850 m), Ethiopia. For comparison, the following Azotobacter strains were added: (1) *A. chroococcum* str. 1 from garden soil of Delft, Holland, (2) *A. chroococcum* str. 5 from margalite soil [22] of Ngawi, Centr. Java, Indonesia, (3) *A. agile* str. 1 from straw-board factory waste water of Groningen, Holland, and (4) *A. vinelandii* str. 9 from a clay soil of Trinidad-Beni, Bolivia.

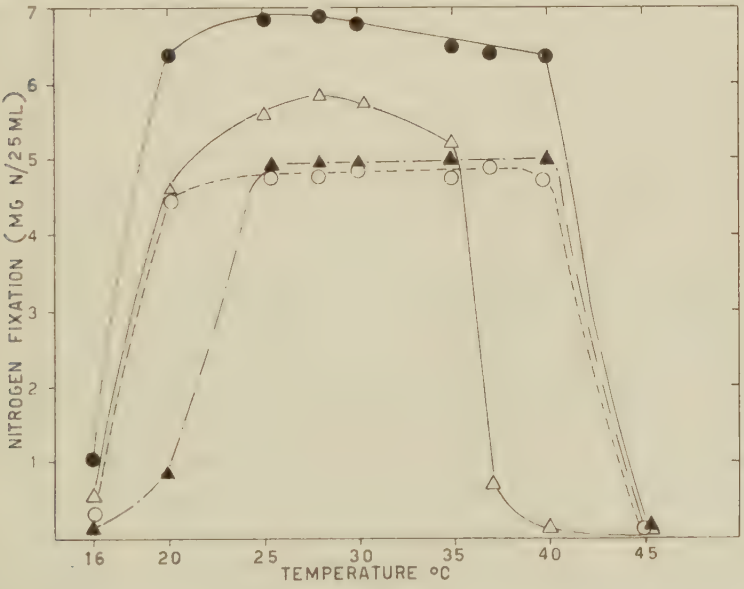

Fig. 2. Nitrogen fixation in relation to temperature in some Azotobacter strains of different origin.

- ● *A. vinelandii* from tropical soil (Bolivia),
- △ *A. chroococcum* from temperate soil (Delft, the Netherlands),
- ▲ *A. chroococcum* from tropical soil (Ngawi, Centr. Java), and
- ○ *A. agile* from waste water of Groningen, (the Netherlands).

The results of these experiments are summarized in Figures 1 and 2. The figures show that the temperature growth range of Beijerinckia is more limited than that of Azotobacter. Both species grew at 16°C, but Beijerinckia did not grow at above 37°C, whereas all Azotobacter strains grew up to 40° or 45°C.

However, there was some strain variability in optimal growth of the Beijerinckia strains. The strain isolated from the Ethiopian highland soil showed an optimal growth range distinctly extended to the lower temperatures of 20° to 30°C. One of the two strains isolated from tropical lowland soils gave optimal nitrogen fixation between 25° and 35°C, while the other strain (str. Bogor) produced a clear optimal-growth peak at 28°C, which is about the same as the mean temperature of the locality of origin.

One *Azotobacter agile* strain and one *Azotobacter vinelandii*, of temperate and tropical origin respectively, did not respond differently to variation in temperature. However, two *Azotobacter chroococcum* strains tested showed a clear adaptation to temperature conditions of the locality from which the soil originated. In the tropical strain, development was poor at 20°C, but optimal growth was still obtained at 40°C, whereas in the strain of temperate origin the reverse was true as evident from Figure 2.

The observation that Beijerinckia was unable to produce growth at 37°C was further checked in fifty other strains of different origin. In order to exclude any differences in environmental conditions, both, Beijerinckia and Azotobacter strains were cultivated on the same agar plates. For this reason, calcium chloride (0.1%) was added to the nitrogen-free glucose medium used for Beijerinckia since Azotobacter needs calcium for optimal growth. The agar plates were divided into sections, on one Beijerinckia was inoculated, on the other Azotobacter. Incubation was at 37°C. At this temperature no Beijerinckia strain produced growth, while all Azotobacter strains showed excellent development. Only in one case using a very large inoculum a poor growth of one Beijerinckia strain (str. Hawaii 3/1) was observed. In the latter strain, cells grown at this temperature were quite different from the original strain, being swollen and of an irregular appearance, while the colony form had also changed, being flat and spreading. Such a modified strain was not very viable, and it was soon lost.

The growth experiments at 16°C were also repeated with many

different strains. All strains showed growth at this temperature, and the cultures were distinctly turbid in 35 days. Incubation for longer periods gave good nitrogen-fixation yields and large glucose consumption.

(b) *Survival at low temperature*

Cells of young, viable Beijerinckia cultures were suspended in either sterile, nitrogen-free 1% glucose medium or in sterile tap water. The suspensions were exposed in test tubes to temperatures of +4°C and −4°C. At regular time intervals the survival of the cells was qualitatively tested by plating some drops of the suspensions on nitrogen-free glucose agar plates followed by incubation at 30°C. Many different Beijerinckia and Azotobacter strains were tested.

In the Beijerinckia strains a storage time of 3 to 4 months at +4°C did not appreciably affect the viability of the cells. After 10 months at this temperature only a few strains which under normal conditions grow slowly or poorly produced fewer colonies or no growth at all on the agar plates. After 16 months at +4°C some relatively fast-growing strains also showed some reduction in quantity of viable cells, but suspensions of the latter strains held for 25 months at this temperature still contained a very appreciable number of living cells.

Storage of Beijerinckia cells at −4°C showed no marked reduction in the number of viable cells after 3 to 4 months. After 7 to 8 months there was some decrease, and after 11 to 12 months of storage at this temperature most suspensions of slow-growing Beijerinckia strains died out. After 20 months most suspensions of the fast-growing strains also did not produce growth, only a very few suspensions giving some isolated colonies on the agar plates. Some agar-slant cultures kept frozen for the same period showed no growth on subcultivation. Hence, a storage time of 20 months at −4°C seemed to be the upper limit of frost-resistance in Beijerinckia.

Azotobacter cells exposed to the above conditions showed some strain and species variability. Transfers from suspensions of *Azotobacter agile* kept for 12 months at +4°C showed no growth, but suspensions of *Azotobacter chroococcum* and *Azotobacter vinelandii* treated in the same way produced luxuriant growth. However, no Azotobacter suspension could be revived after 12 months at −4°C.

3 RESISTANCE AGAINST DROUGHT

(a) *Experiments with dried suspensions and agar slants*

Suspensions of Beijerinckia were placed over $CaCl_2$ in a desiccator and completely dehydrated. After subsequent wetting, cell survival was tested qualitatively by plating. It was observed that most Beijerinckia strains can easily resist this treatment for periods of one to several weeks. However, there was some strain variability: some more susceptible strains showed less growth or no growth at all after being held under these conditions for 3 to 5 weeks.

Less rigorous desiccation experiments were performed with agar-slant cultures of Beijerinckia. These cultures were allowed to dry at room temperature and kept in this state at normal room humidity for more than 7 to 8 months. At the end of this period, of the 135 different strains tested, only four (3 per cent) did not produce growth when subcultured.

(b) *Survival in soils kept under air-dry conditions*

The survival of Beijerinckia was quantitatively followed in both acid and alkaline European soils and in an acid tropical lateritic soil. From the latter soil Beijerinckia was isolated.

The European soils used were obtained from experimental plots laid down in the garden of the Laboratory of Microbiology at Wageningen (Netherlands). The soils were originally the same acid sandy soil of light texture. Two plots were tested: one treated with lime and phosphate, the other untreated. In the limed plot alkaline soil conditions had developed. This soil contained abundant Azotobacter.

Two hundred and fifty gram soil of the different samples were placed in Neubauer glass jars and inoculated with a dense suspension of Beijerinckia cells. All experiments were conducted using duplicate sets of samples. In one set the soils were held at optimal moisture content (10% of dry weight), in the other set water loss was not replaced and these soils soon reached air-dry condition. The soils were incubated in complete darkness in a constant-temperature room at 25°C for 90 days.

The number of viable cells was counted by a plating procedure which was a modification of Winogradsky's silica-gel technique [40]. The soil was removed from the jars and spread in a thin layer on paper until air dry which took about 2 hours at 30°C. It was then powdered with a sterile

spatula, and various quantities (2 to 50 mg) of soil were weighed out accurately and distributed as uniformly as possible over the surface of large (20 cm diameter) agar plates containing acid, (pH 5.0) nitrogen-free glucose agar. Cell counts were made after 14 to 21 days. All soils were tested in 5 replicates. The entire experiment was carried out twice. The average number of bacteria, calculated per gram of soil, is given.

TABLE 6

Survival of Beijerinckia in soils kept for 90 days at optimal soil moisture content and under air-dry conditions

Soil type	Soil treatment	Dressing	Soil pH	Number of bacteria per g air-dry soil	
				Initial	Final
Tropical laterite soil * . . .	Wet	not treated	4.8	750,000	313,000
	Dry	,,	,,	,,	130,000
European sandy soils **					
Experimental plot 18 . .	Wet	0 Ca, 0 P	5.9	570,000	184,000
	Dry	,,	,,	,,	10,000
Experimental plot 13 . .	Wet	+ Ca, + P	7.3	520,000	409,000
	Dry	,,	,,	,,	6,000

* From Bogor, W. Java, Indonesia.

** From the experimental plots, each 42 m², of the Laboratory of Microbiology at Wageningen, Holland. The plots received annually a basic dressing of Chilean nitrate (1.5 kg/42 m²) and potassium sulphate (1.25 kg/42 m²). Plot 13 was dressed in addition with calcium carbonate (60 kg/42 m²) and superphosphate (2 kg/42 m²), while plot 18 received neither calcium nor phosphate.

Table 6 shows that in the tropical and European soils the rate of survival of Beijerinckia was higher under moist than under dry conditions. The tropical soil showed a higher rate of survival than did the European. The latter result agrees with the observation that Beijerinckia is rather persistent in tropical lateritic soils stored in air-dry condition at room temperature, since after 5 years Beijerinckia could still be isolated from these soils. However, most of the latter isolations proved to be capsule-producing Beijerinckia strains (see Ref.[2]). Thus, this characteristic gave it an increased resistance to drought.

In European soils in humid condition the alkaline sample had a survival rate three times higher than that of the acid soil sample. This result is rather surprising since in the tropics Beijerinckia occurs mainly in more or less acid soils and does not need calcium for development. This decrepancy will be dealt with extensively in a separate paper.

4. THE INFLUENCE OF SOIL PH

It is a well-known fact that the occurrence of Azotobacter in soil requires a pH value of 6.0 or higher [3 9]. Only in a few cases have abnormal Azotobacter strains been isolated which were resistant to acid reaction [14 37]. Since Beijerinckia has been frequently found in acid soils, a study was made to ascertain the relationship between occurrence of Beijerinckia and soil pH.

Beijerinckia is almost entirely absent in soils of the temperate region whatever their pH value. For this reason this survey was restricted to tropical soils. A classification of the soils was made according to their pH and in each pH range the percentage of samples positive for Beijerinckia or Azotobacter was determined. The results of this survey are given in Table 7.

It will be seen from this table that the largest percentages of Beijerinckia-positive soils were found in the pH ranges 5.5 to 5.9 (74.2 per cent) and 5.0 to 5.4 (64.3 per cent). At higher and lower pH values a gradual decrease in the percentages of Beijerinckia-positive soils was observed; below pH 4.0 and above pH 7.4 Beijerinckia was entirely absent. Azotobacter was absent in all soils with a pH below 6.0. The percentage of Azotobacter-positive soils regularly increased from pH 6.0 to pH 7.5, and all soils above a pH 7.5 without exception contained Azotobacter.

It is remarkable that the decrease in the percentage of Beijerinckia-positive soils with increasing pH coincided more or less with an increase of Azotobacter-positive soil samples. Above pH 7.4 Beijerinckia had completely disappeared, whereas all soils contained Azotobacter. This suggests that an alkaline reaction is not *per se* unfavourable for the growth of Beijerinckia, but due to its intrinsic slow growth rate this bacterium may not be able to compete effectively with Azotobacter occurring in the same soil.

This suggestion was tested in growth experiments with Beijerinckia strains in pure culture in media with varying pH values. A number of strains isolated from acid and alkaline soils were tested. Since most results showed about the same tendency only a few typical experiments are reported.

The Beijerinckia strains were grown in the nitrogen-free glucose medium mentioned previously. The medium was pipetted in 25-ml aliquots into 100-ml erlenmeyer flasks and adjusted with varying amounts of 0.1 *N* NaOH or 0.1 *N* H_2SO_4 to pH values ranging between pH 2.5 and 10.0.

TABLE 7

The pH distribution of the 148 tropical soils tested and the percentage Beijerinckia- and Azotobacter positive soils in each pH range

Number of	pH range												Total
	3.0–3.4	3.5–3.9	4.0–4.4	4.5–4.9	5.0–5.4	5.5–5.9	6.0–6.4	6.5–6.9	7.0–7.4	7.5–7.9	8.0–8.4	8.5–9.0	
Soils tested	1	2	11	18	28	31	19	14	9	11	3	1	148
Beijerinckia-positive soils	0	0	2	8	18	23	10	7	3	0	0	0	71
Percentage in each pH range	0	0	18.2	44.4	64.3	74.2	52.6	50.0	33.3	0	0	0	48.0
Azotobacter-positive soils	0	0	0	0	0	0	6	8	8	11	3	1	37
Percentage in each pH range	0	0	0	0	0	0	31.6	57.1	88.9	100	100	100	25.0

TABLE 8

The percentage of Beijerinckia positive soils in relation to soil group and pH range for the 148 tropical soils tested

Number of	pH range												Total
	3.0–3.4	3.5–3.9	4.0–4.4	4.5–4.9	5.0–5.4	5.5–5.9	6.0–6.4	6.5–6.9	7.0–7.4	7.5–7.9	8.0–8.4	8.5–9.0	
Soils tested	1	2	11	18	28	31	19	14	9	11	3	1	148
Lateritic soils, tropical Red Earths and ferruginous loams . . .	1	2	8	17	24	22	10	6	0	2	0	0	92
Other soil types	0	0	3	1	4	9	9	8	9	9	3	1	56
Beijerinckia-positive soils	0	0	2	8	18	23	10	7	3	0	0	0	71
Positive lateritic soils .	0	0	2	8	18	20	6	4	0	0	0	0	58
Percentage in each pH range	0	0	25.0	47.1	75.0	90.9	60.0	66.7	0	0	0	0	63.0
Positive other soil types	0	0	0	0	0	3	4	3	3	0	0	0	13
Percentage in each pH range	0	0	0	0	0	33.3	44.4	37.5	33.3	0	0	0	23.3

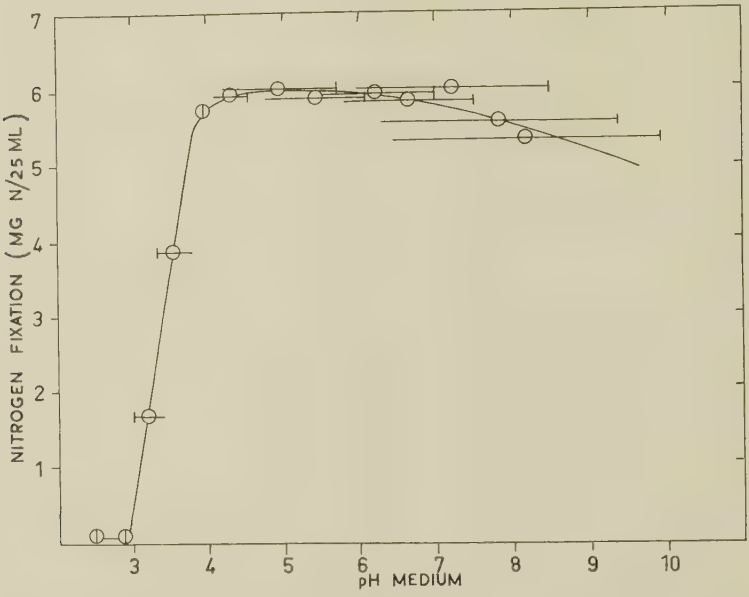

Fig. 3. Nitrogen fixation of *Beijerinckia indica* str. Hawaii 2/1 isolated from an acid (pH = 5.5) latosol in relation to the pH of the medium. (Vertical lines indicate initial pH values, horizontal lines the pH change of the medium during growth).

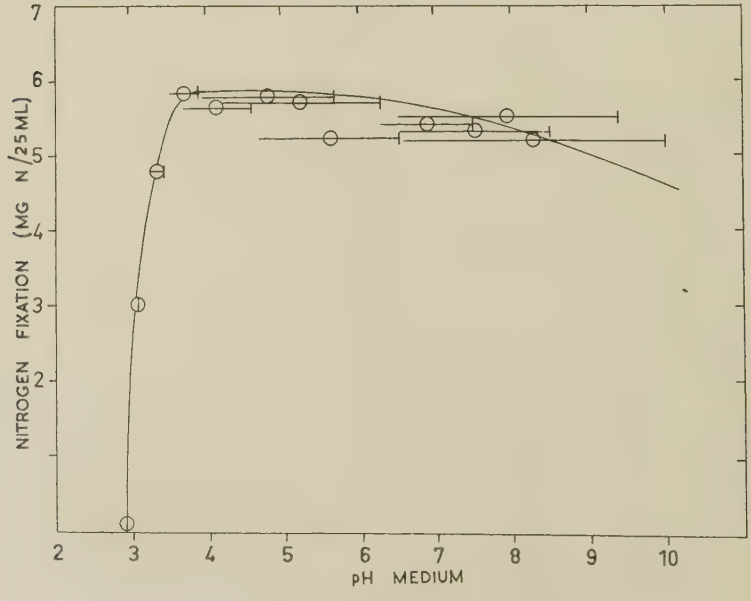

Fig. 4. Nitrogen fixation of *Beijerinckia indica* str. Hongkong 2/2 isolated from an alkaline (pH = 7.4) sandy soil in relation to the pH of the medium. (Vertical lines indicate initial pH values, horizontal lines the pH change of the medium during growth).

The flasks were incubated under stationary culture conditions in air for 35 days at 30°C. The medium did not stay at constant pH during growth, but it was not buffered with phosphate buffer since phosphate at high concentrations was inhibitory for growth. Organic buffers were not used because they influenced nitrogen fixation.

Fixed nitrogen, estimated by the micro-Kjeldahl procedure as modified by Yuen and Pollard [41], was used as a measure of growth. The results, average values of duplicate experiments, are plotted in Figures 3, 4, and 5.

The experiments reported were conducted with the following *Beijerinckia indica* strains: (1) str. Hawaii 2/1 from an acid (pH 5.5) "Low Humic Latosol" of Poamoho, Oahu, Hawaii Islands (Figure 3) and (2) str. Hongkong 2/2 from an alkaline (pH 7.4) sandy soil from Yung Shu Wan, Lamma Islands, Hongkong (Figure 4). The results with a Beijerinckia strain, *B. mobile* str. 1 isolated from an extreme acid (pH 4.5) laterite from Bogor, W. Java, Indonesia, are given in Figure 5.

As obvious from a comparison of Figures 3 and 4, the strains from the acid and alkaline soil showed about the same pattern of nitrogen fixation in relation to pH medium. Optimal growth was already reached at pH 4.0 and at increasing pH values up to pH 9.0 to 10.0 there was only a small reduction in nitrogen fixation. The strain from acid soil used was obtained from a moderately acid (pH 5.5) soil, but isolations from far more acid soils showed the same pattern. The strain from alkaline (pH 7.4) soil was isolated from the most alkaline soil containing Beijerinckia.

In only one Beijerinckia strain was a different pattern obtained. This strain, isolated from a very acid (pH 4.5) laterite, showed optimal nitrogen fixation at about pH 3.9, and a considerable drop in nitrogen fixation to alkaline reaction which at pH 9.0 gave about 50 per cent reduction (Figure 5).

The outcome that in the great majority of the Beijerinckia strains tested nitrogen fixation was not much reduced at relatively high pH values corroborated the suggestion made above, that the absence of Beijerinckia in soils above a pH 7.4 is not caused by a pH effect, but must be due to other factors.

As is evident from the results, there was a considerable pH shift of the medium during Beijerinckia growth. At more alkaline reactions acid was produced and at very acid reactions the pH of the medium increased. At a certain intermediate pH value, in spite of very good growth, neither acid nor alkali was produced. The latter pH value was in most strains tested between pH 3.0 and 4.0 (see

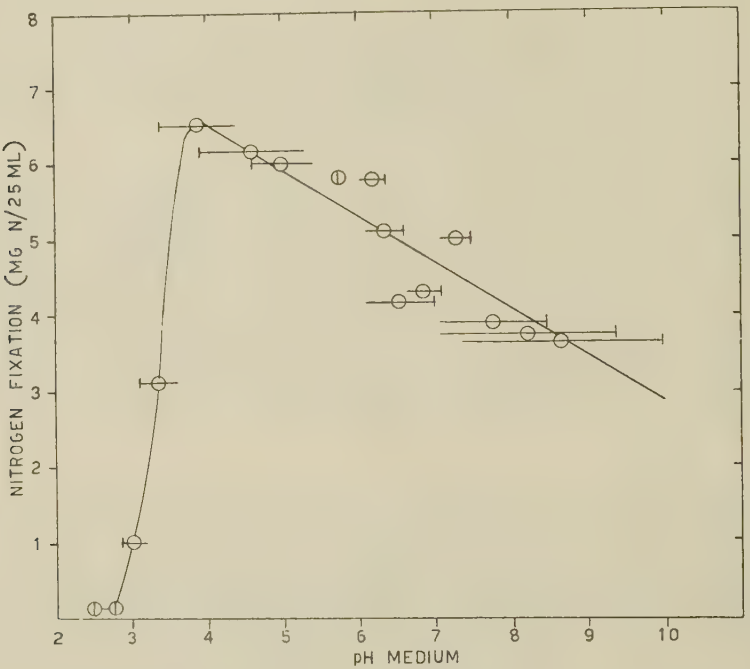

Fig. 5. Nitrogen fixation of *Beijerinckia mobile* str. 1 isolated from a very acid (pH = 4.5) laterite of Bogor (W. Java) in relation to the pH of the medium. (Vertical lines indicate initial pH values, horizontal lines the pH change during growth).

Figures 3 and 4), but in the aberrant *Beijerinckia mobile* strain at pH 5.8 (see Figure 5).

The medium of the cultures grown at more alkaline reaction were analysed for organic acids.

The bacteria were removed from the medium by centrifugation using a Cepa continuous-flow centrifuge. The medium was then deproteinized by $Zn(OH)_2$ addition (Neish [26]) and recentrifugation. After acidifying the medium with H_2SO_4, the volatile acids were collected by steam distillation and the non-volatile acids by ethyl ether extraction. Both mixtures of acids were analyzed by the use of one-dimensional paper chromatography according to the method of Kennedy and Barker [16].

In addition, the fraction containing the non-volatile acids was separated by two-dimensional paper chromatography following a modification of Munier's [25] method, *viz* first elution with ethanol—ammonia—water (80 : 4 : 16), second elution with *n*-butanol—formic acid—methylbenzoate—water (50 : 20 : 50 : 1.5). The spray solution suggested by Heitefusz [11] was used to indicate the spots.

Acetic acid was found to occur as the only volatile acid. It was the major component of the acids produced in the media, since the non-volatile acid detected in the ether fractions was only present in very small quantities. The latter unknown acid gave after elution with the alkaline solution a spot with a $R_f = 0.57$, *i.e.* between lactic and fumaric acid used as standards.

In no case was lactic acid found in the culture medium of a great number of Beijerinckia strains tested. This in contrast to results of Kauffmann and Toussaint [15] who described a new *Beijerinckia* species, *B. lacticogenes*, showing lactic acid production. In the latter strain, however, which was kindly placed at our disposal by Dr. H. L. Jensen (Lyngby, Denmark), under our experimental conditions only acetic acid and no lactic acid was found in medium.

The alkaline substance or substances produced by Beijerinckia at very acid reaction have not yet been identified.

5. THE INFLUENCE OF SOIL TYPE

To investigate the influence of soil type, the 148 tropical soils examined were classified in two main groups: I lateritic loams, related Red Earths, and ferruginous loams; and II other soil types comprising littorial sands, sandy soils, margalite (Mbuga) soils, and other clays of illuvial or alluvial origin. Ninety-two (62 per cent) of the tropical soils tested belonged to the first soil group and 56 (38 per cent) to the latter. Table 8 shows that 58 (63 per cent) of the 92 lateritic soils, but only 13 (23 per cent) of the 56 other soils tested contained Beijerinckia.

Thus, in addition to the fact that lateritic soils are far more frequently distributed in the tropics than any other soil type, Beijerinckia shows a distinct preference for this soil type. As evident from the pH distribution of the lateritic soils, 91 per cent of the lateritic soils of pH 5.5 to 5.9 contained Beijerinckia, and its distribution in the latter soils was only limited by very acid or alkaline reaction. No lateritic soil having a pH below 4.0 contained Beijerinckia.

Of the other soil types containing Beijerinckia there were some alluvial soils, a few illuvial soils, cultivated garden soils, typical forest soils, and the volcanic ash soils of Japan. The occurrence in some forest soils was interesting as this organism was present in

these soils on the litter fragments of the litter layer. No particular relation between the distribution of Beijerinckia and vegetation was, however, evident, since Beijerinckia was found equally readily and often in very great numbers in some soils of exposed sites, open grassland or savannah. Forty-seven per cent of the Beijerinckia-positive tropical soils were uncultivated soils and 53 per cent cultivated soils.

As is obvious from the preceding, soil type and soil pH mainly govern the distribution of Beijerinckia in soils. This was shown clearly by examining the distribution of Beijerinckia in some regions of Asia and Africa which have very pronounced soil type differences within short distance. In the latter regions leaching may develop in catenary succession acid lateritic soils on the hills and alkaline illuvial soils in the valleys. Beijerinckia occurred abundantly in the first soil type irrespective of cover and was almost absent in the latter. An appreciable lime content seemed to be especially inhibitory for the distribution of Beijerinckia, since this organism was nearly entirely absent in soils containing calcium carbonate concretions. In the latter soils Beijerinckia was completely replaced by Azotobacter.

Due attention should be paid to the distribution of Beijerinckia in the volcanic ash soils of Japan. Suto [35] [36] has already reported the occurrence of Beijerinckia in a volcanic ash soil of Sendai (Lat. 38° N.). A number of soils of other parts of Japan (Tokyo, Lat. 35° N. and Nikko National Park, Lat. 37° N.) examined in the present study all showed plenty of Beijerinckia. The occurrence of Beijerinckia in volcanic ash soils of Japan must therefore be connected with this particular soil type. On the other hand, the distribution of Beijerinckia outside the tropics in South Africa coincided more or less with the distribution of lateritic soils which have developed under special climatological conditions ("Mist-belt") in northern Transvaal. This observation is in good agreement with the conclusion that lateritic soils form a preferential environment for Beijerinckia.

DISCUSSION

The present survey covering the examination of about 400 soil samples of temperate and tropical origin conclusively showed the distribution of Beijerinckia to be mainly in tropical soils. In tropical

soils Beijerinckia is found far more frequently than Azotobacter: of the 148 tropical soils tested 48 per cent contained Beijerinckia and 25 per cent Azotobacter.

Of the 244 non-tropical soils examined, 9 per cent contained Beijerinckia and 38.5 per cent Azotobacter. The only non-tropical soils containing Beijerinckia were obtained from the following localities: Medvea and Kostrena (Lat. 45° N.) near Rijeka (Fiume) in Yugoslavia, Cape Town (Lat. 34° S.) and in particular the "Mist-belt" region (Lat. 23–25° S.) of northern Transvaal in South Africa, Koelpura (Himalaya Mountains, Lat. 30° N.) in northern India, and Tokyo (Lat. 35° N.) and Nikko National Park (Lat. 37° N.) in Japan. The latter confirms a recent observation made by Suto [35 36] that Beijerinckia occurred in a volcanic ash soil at Sendai (Lat. 38° N.) in Japan. The Beijerinckia-positive soils from Japan examined in the present survey were also mostly volcanic ash soils. The distribution of Beijerinckia in South Africa was mainly restricted to the lateritic soils of the "Mist-belt" region, whereas the only European soils found to be positive for this organism were "terra rossa" soils, a weathering product of lime stone.

Experiments with some Beijerinckia and Azotobacter strains of different origin showed a more narrow growth temperature range in Beijerinckia compared with Azotobacter. All Beijerinckia strains and most Azotobacter strains tested produced some growth at 16°C, but in great contrast to Azotobacter, no Beijerinckia strain grew at 37 C, whereas most Azotobacter strains showed luxuriant development at 37°C and some even at 40°C.

In spite of the fact that Beijerinckia shows no particular adaptation to the higher temperatures of tropical regions, there was within its growth limits in relation to temperature some strain variability corresponding to local temperature conditions. Such an adaptation was also noted in two *Azotobacter chroococcum* strains isolated from temperate and tropical soil, but one *Azotobacter agile* strain from temperate origin and one tropical *Azotobacter vinelandii* strain did not respond differently to variation in temperature. High optimum temperatures in Azotobacter strains from hot climates have also been reported by Greene [10] and Pathak [27].

The fact that Beijerinckia could survive 25 months at +4°C and 20 months at −4°C indicates that low temperature and frost cannot be responsible for the absence of Beijerinckia in the temperate

region. Comparative experiments performed with Azotobacter showed that it is far less resistant against low temperature and frost than Beijerinckia. In addition, the soil survey revealed the occurrence of Beijerinckia in some tropical soils collected at very high altitudes, *viz* in soils of Kenya and Tanganyika Highlands (2100 to 2500 m), Ethiopia (2500 to 2800 m) and Java (1500 to 3000 m). Moreover, in Japan (Nikko National Park, 1300 to 1500 m) Beijerinckia was obtained from a soil which was then covered with melting snow.

Beijerinckia was also observed to be rather resistant to desiccation, since most strains resisted desiccation over $CaCl_2$ for several weeks, confirming an earlier observation by Tchan[38]. From 135 agar-slant cultures desiccated and kept for 7 to 8 months under air-dry condition at room temperature, only 3 per cent could not be revived.

Nevertheless, compared to data given in the literature for *Azotobacter chroococcum* (see Stapp and Ruschmann [33]) Beijerinckia was found to be more susceptible to the effects of drought. When cultures of Beijerinckia were kept in soil under air-dry conditions for 90 days, a deleterious effect on viability was found to occur. This was particular the case in an acid European soil; in tropical lateritic soils this effect was much less pronounced. Storage under optimal moisture conditions in the European soil also gave a considerable decrease of viable Beijerinckia cells. However, when the soil had been treated with $CaCO_3$ and phosphate, as a result of which its pH had increased from pH 5.9 to 7.3, survival was much more favourable. This effect is unexpected, because Beijerinckia may be found preferentially in the more or less acid and lime-deficient soils of tropical regions.

It was observed that under natural conditions most Beijerinckia-positive soils occurred at pH 5.5 to 5.9 (74.2 per cent) and 5.0 to 5.4 (64.3 per cent). From these pH values, there was a sharp decline in both, acid and alkaline directions. No soil below pH 4.0 and above pH 7.4 contained Beijerinckia.

It is suggested that the disappearance of Beijerinckia in soils with alkaline reaction might be due to a competition with Azotobacter, since the gradual decrease in the percentage Beijerinckia-positive soils at alkaline pH values coincided with an increase in the percentage of Azotobacter-positive soils.

Experiments with pure Beijerinckia cultures growing in media

with varying pH values showed in most strains, irrespective of whether they have been isolated from acid or alkaline soils, a rather broad optimal growth range in relation to pH. Optimal nitrogen fixation was already reached at pH 4.0 and did not appreciably decline at pH 9.0 to 10.0. This observation supports the suggestion that the absence of Beijerinckia in soils above pH 7.4 is not due to a pH effect. Only one Beijerinckia strain, *B. mobile* str. 1, isolated from a very acid laterite (pH 4.5), showed optimal nitrogen fixation at pH 3.9 and about 50 per cent reduction in growth at pH 9.0.

During growth of Beijerinckia the pH value of the culture medium did not remain constant. At very acid reaction the pH was increased, but in more alkaline media there was a pH drop during growth. At an intermediate pH value (mostly at pH 3.0 to 4.0, except in the above-mentioned *B. mobile* strain at pH 5.8) no change of reaction occurred in spite of very good growth. The acids produced in a relative alkaline nutrient medium were found to be acetic acid and an unidentified non-volatile acid in very small quantities. The production of acetic acid by Beijerinckia has already been reported by Quinnell [28] for the type strain.

Next to pH, soil type seemed to be of prime importance for the distribution of Beijerinckia. Of 148 tropical soils tested, 62 per cent belonged to lateritic and related soil types and 38 per cent to other soils. Of the lateritic soils, 63 per cent were Beijerinckia-positive, whereas only 23 per cent of the other soil types contained Beijerinckia. These results indicate a preponderant preference of Beijerinckia for soils of the lateritic type. In fact nearly all lateritic soils with suitable reaction contained Beijerinckia in very great numbers. Only the very acid reactions sometimes developed in this soil type limited its distribution in these soils. The observation that Beijerinckia occurs mainly in the lateritic soils of tropical regions confirms earlier observations made by Kluyver and Becking [17] and Becking [2]. This explains to a certain extent the preference of nitrogen-fixing bacteria of the genus *Beijerinckia* for tropical regions, since laterite formation is only clearly displayed in the tropics (cf Russel [31]).

It is well known that laterization leads to an accumulation of iron and aluminium in the upper soil layers and to a leaching out of silica, potassium, sodium, and, more particularly, calcium. The

extreme poverty in calcium of lateritic soils may be connected with the observation made by Jensen [12 13] that Beijerinckia, in contrast to Azotobacter, does not require calcium for its development. It might be suggested that the distribution of Beijerinckia in tropical soils was related to the particular mineral composition of lateritic soils. Therefore, a number of experiments on mineral nutrition of Beijerinckia and Azotobacter have been carried out. The results of these experiments will be reported in a subsequent paper.

SUMMARY

A survey was made of the distribution of the nitrogen-fixing bacteria of the genus *Beijerinckia* (syn. *Azotobacter indicum* Starkey and De) in soils. It was found that this organism occurs mainly in soils of tropical regions. In these regions Beijerinckia is far more frequently distributed than Azotobacter. Of the 148 tropical soils examined, 48 per cent contained Beijerinckia and 25 per cent Azotobacter. Of the 244 non-tropical soils tested 9 per cent contained Beijerinckia and 38.5 per cent Azotobacter. The only non-tropical soils which were found to contain Beijerinckia originated from Southern Europe (Medvea and Kostrena near Rijeka in Yugoslavia), South Africa (northern Transvaal and Cape Town), northern India (Koelpura, Himalaya Mts.), and Japan (Tokyo, Nikko National Park).

The tropical distribution of Beijerinckia was not due to an adaptation to the higher temperatures of the tropics. Comparative experiments with Beijerinckia and Azotobacter strains revealed a narrower temperature growth range in Beijerinckia than in Azotobacter. Beijerinckia strains (50 strains tested) and most of the Azotobacter strains produced some growth at 16°C, but – in great contrast to Azotobacter – no Beijerinckia strain grew at 37°C. Nevertheless, within the temperature growth range of Beijerinckia some strain variability was noted, correlated with local temperature conditions. The same phenomenon was observed in two *Azotobacter chroococcum* strains, but not in one *Azotobacter agile* and one *Azotobacter vinelandii* of temperate and tropical origin.

Beijerinckia was found to be exceedingly cold and frost resistant; cell suspensions of most strains survived a storage time of 25 months at +4°C and 20 months at −4°C. Therefore, low temperature and frost cannot be responsible for the absence of this bacterium in the temperate zone.

Beijerinckia was rather resistant to desiccation, but when this organism was kept in soil under air-dry condition a deleterious effect on survival was noted, in particular in acid European soils. The latter effect was far less apparent in the lateritic soils which form its natural habitat. There was also a pronounced decrease in the number of viable cells under optimal moisture condition in acid European soils. However, the application of lime to this acid soil, which increased its pH value, had a very favourable effect on the survival of Beijerinckia in this soil.

Under natural conditions the highest percentage of Beijerinckia-positive soils (related to the number of soils in each pH range) was found in the pH range 5.5 to 5.9 (74.2 per cent) and 5.0 to 5.4 (64.3 per cent). No soil with a pH below 4.0 and above 7.4 contained Beijerinckia. Since the latter soils without exception contained Azotobacter, it is suggested that the absence of Beijerinckia might be due to competition with Azotobacter and not to alkaline conditions itself. This view is supported by the observation that most Beijerinckia strains (irrespective of whether they have been isolated from acid or alkaline soils) reached optimal nitrogen fixation at pH = 4.0 and gave only a very small reduction in nitrogen fixation with very alkaline reaction, pH 9.0 to 10.0. Only in one strain (*B. mobile* str. 1) was there about 50 per cent reduction of growth at pH 9.0.

During growth of Beijerinckia in nitrogen-free media the reaction of the medium changed: at very acid reaction the pH of the medium increased, whereas in more alkaline media, acid was produced. Acid production was increased at increasing pH values. At an intermediate pH value (mostly pH 3.0 to 4.0, but in one case pH 5.8) neither acid nor alkali was produced in spite of very good growth. The acids produced were acetic acid and an unidentified non-volatile acid which was present in very small quantities.

Next to pH, soil type seemed to be of prime influence on the distribution of Beijerinckia. Sixty-two percent of the tropical soils tested belonged to the lateritic soil type. Of the lateritic soils 63 per cent were Beijerinckia-positive and only 23 per cent of the other soil types. This observation provides to a partial explanation for the mainly tropical distribution of this organism since laterite formation is only found in the tropics. It is possible that Beijerinckia is adapted to the characteristic chemical composition of lateritic soils. This question was studied and the results will be dealt with in a separate paper.

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LETTER TO THE EDITORS

Zur Möglichkeit der Düngung mit Rohphosphat

Früher [1] wurde gezeigt, daß der Hydroxylfluorapatit, ein isomorphes Gemisch von Hydroxylapatit und Fluorapatit mit höchstens 70% Fluorapatit das Mineral der Rohphosphate darstellt, und reiner Fluorapatit sowie Chlor-, Carbonat- und Sulfatapatit bei Gegenwart von Wasser nicht existenzfähig sind. Ob dieser Hydroxylfluorapatit als Mineral in einem Boden vorkommt, hängt davon ab, ob er im Gleichgewicht mit den in diesem Boden vorhandenen Austauschersubstanzen Aluminium- und Eisen- Hydroxyd- Silikat- Phosphat- Humat existenzfähig ist. Ist diese Existenzfähigkeit des Hydroxylfluorapatits in einem Boden nicht gegeben, so würde eine Zufuhr von Rohphosphat zu diesem Boden eine düngende Wirkung haben. Im Folgenden soll daher das Gleichgewicht des Hydroxylfluorapatits mit Aluminium- und Eisen- Hydroxyd- Phosphat untersucht werden.

Wie vor kurzem [3] beschrieben, erfolgt die Darstellung von Aluminium- und Eisen- Hydroxyd- Phosphat durch gleichzeitige Zugabe von Aluminium- oder Eisen- und Phosphationen in verschiedenen Mengenverhältnissen zu siedendem Wasser unter Konstanthaltung des pH-Wertes. Der Hydroxylfluorapatit wird in ähnlicher Weise durch gleichzeitige Zugabe von Calcium-, Phosphat- und Fluorionen in solchen Mengenverhältnissen, wie sie im Hydroxylfluorapatit vorliegen, zu siedendem Wasser unter Konstanthaltung des pH-Wertes dargestellt [1]. Werden daher Calcium- Phosphat- und Fluorionen im Apatitverhältnis sowie gleichzeitig Aluminium- oder Eisenionen in verschiedenen Mengenverhältnissen in bezug auf die zugesetzten Phosphationen zu siedendem Wasser unter Konstanthaltung des pH-Wertes zugesetzt, so erfolgt die gleichzeitige Ausfällung von Hydroxylfluorapatit und Aluminium- und Eisen-Hydroxyd- Phosphat, wobei wegen der Entstehung aus wäßriger Lösung sofort Gleichgewichtseinstellung erfolgt. Die nach der Ausfällung in Lösung befindliche Calciummenge zeigt an, wie weit das Gleichgewicht auf seiten des Hydroxylfluorapatits oder des Aluminium- bezw. Eisen- Hydroxyd- Phosphates liegt.

Zur Ausführung entsprechender Versuche wurden in mehreren Ansätzen aus je einer Bürette eine Lösung von Hydroxylfluorapatit in Salzsäure sowie verschiedenen Mengen angesäuerte Aluminium- oder Eisensalzlösung in 1 l siedendes Wasser tropfen gelassen, dessen pH-Wert durch eine eintauchende Glaselektrode * gemessen und durch gleichzeitiges Zutropfen von Natron-

* Für die Temperatur von 100° war eine Neueichung der Glaselektrode erforderlich.

TABELLE 1

Gemeinsame Ausfällung von Hydroxylfluorapatit und von Aluminium- oder Eisen-Hydroxyd-Phosphat

Zugesetzt wurden je Ansatz:
0,1 mMol (= 100 mg) Hydroxylfluorapatit, enthaltend 1,0 mMol Ca und 0,6 mMol P, gelöst in Salzsäure,
0,6, 1,2, 2,4 und 4,8 mMol Aluminium- oder Eisensalz, gelöst in Salzsäure

Al : P oder Fe : P	pH-Wert während der Ausfällung	Gelöstes Ca nach der Ausfällung (mMol)	Hydroxylfluor-apatit im Nieder-schlag (mMol)	Gelöstes Phosphat (mg P_2O_5 je Liter)
Al : P				
1 : 1	7,0	0,10	0,090	0,2
1 : 1	6,0	0,18	0,082	0,4
1 : 1	5,0	0,21	0,079	0,7
2 : 1	7,0	0,16	0,084	0,2
2 : 1	6,0	0,24	0,076	0,5
2 : 1	5,0	0,48	0,052	0,8
4 : 1	7,0	0,30	0,070	0,2
4 : 1	6,0	0,82	0,018	0,4
4 : 1	5,0	0,98	0,002	0,2
8 : 1	7,0	0,41	0,059	0,2
8 : 1	6,0	0,98	0,002	0,2
8 : 1	5,0	0,99	0,001	0,1
Fe : P				
1 : 1	7,0	0,18	0,082	0,2
1 : 1	6,0	0,27	0,073	0,4
1 : 1	5,0	0,39	0,061	0,8
2 : 1	7,0	0,24	0,076	0,2
2 : 1	6,0	0,37	0,063	0,3
2 : 1	5,0	0,50	0,050	0,7
4 : 1	7,0	0,33	0,067	0,2
4 : 1	6,0	0,60	0,040	0,4
4 : 1	5,0	0,99	0,001	0,2
8 : 1	7,0	0,41	0,039	0,2
8 : 1	6,0	0,98	0,002	0,1
8 : 1	5,0	0,99	0,001	0,1

lauge in den verschiedenen Ansätzen verschieden hoch (zwischen pH 5,0 und 7,0) gehalten wurde. Nach Beendigung der Ausfällung wurde nach Abkühlen und Filtrieren der Phosphatgehalt und der Calciumgehalt in der wäßrigen Phase bestimmt.

Aus Tabelle 1 ist ersichtlich:

a. Hydroxylfluorapatit wird bei der gemeinsamen Ausfällung mit Aluminium- und Eisen-Hydroxyd-Phosphat um so weniger gebildet, je saurer die Reaktion und je größer das Verhältnis Al : P oder Fe : P ist,

b. Oberhalb bestimmter Wasserstoffionenkonzentrationen und bestimm-

ter Verhältnisse Al : P oder Fe : P ist kein Hydroxylfluorapatit vorhanden.

c. Bei Vorhandensein von Hydroxylfluorapatit sind die seinem Löslichkeitsgleichgewicht entsprechenden Phosphatkonzentrationen [2] annähernd eingestellt.

Diese Befunde könnten die bekannten landwirtschaftlichen Erscheinungen klären, daß im Allgemeinen eine Zufuhr von Rohphosphat auf sauren, humosen Böden (mit geringem Gehalt an Aluminium und Eisen) düngewirksam ist, daß aber auch auf neutralen, stark phosphorsäurearmen Mineralböden (mit geringen Molarprozenten an Phosphat in den Aluminium- und Eisen--Hydroxyd-Silikat-Phosphat-Humaten) eine Düngewirkung von Rohphosphat gefunden wurde.

WERNER RATHJE,
Berlin N 65, Amrumer Strasse 32

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* Die Versuche zu dieser Arbeit sowie zu den drei früheren, in dieser Zeitschritf erschienenen Arbeiten wurden im Institut für Pflanzenernährung und Bodenbiologie der Technischen Universität Berlin ausgeführt.

LETTER TO THE EDITORS

A Comparison of the Nodulation of Diploid and Tetraploid Varieties of Red Clover Inoculated with Different Rhizobial Strains

The basic chromosome number of the Trifolieae, which are an ancient stock of the Papilionaceae is $n = 8$, but within the tribe there exist also hypnoploids where $n = 7$. *Trifolium pratense* belongs to the latter group. Within recent years, however, artificially induced tetraploid varieties have been introduced agriculturally. As the relationship between the host legume and the root-nodule organism is an extremely variable one, where any change in either the plant *or* the rhizobium may result in optimal symbiotic activity not being achieved, it is important to know whether all strains which cause effective nodulation of diploid plants will be equally effective on tetraploids. Nillson and Rydin [6] stated that no specific rhizobial strain was necessary for optimal fixation of nitrogen by the Swedish tetraploid red clover strain U 036, but, on the other hand, their nitrogen analyses showed a higher nitrogen content in diploid plants than in tetraploid plants. Also, the number of nodules found on diploid plants was greater than that on the tetraploids, over a given period. In the present experiment, three different strains of *Rhizobium trifolii* were tested for performance on a diploid and a tetraploid variety respectively of *Trifolium pratense*.

Materials and methods

The test plants were (a) a diploid variety of *Trifolium pratense* var. Chilean red, where $2n = 14$, and (b) a tetraploid variety of *T. pratense*, kindly supplied by Dr. A. Evans, of the Welsh Plant Breeding Station, Aberystwyth. Three different strains of *Rh. trifolii*, F, SA, and H, were tested for performance on both the diploid and tetraploid plants, and a control of uninoculated diploid and tetraploid plants was also set up.

The seeds were surface sterilised by the method of Thornton [12], and germinated on water-agar plates for 48 hours. They were then transferred aseptically to a 2-day-old inoculum of the appropriate rhizobial strain in yeast-mannitol water for thirty seconds, and planted in jars plugged with cotton wool and containing 150 ml nitrogen-free mineral-agar medium made according to the formula of Bhaduri [2]. There were 3 plants per jar, and 16 jars per treatment. The plants were kept in a cool greenhouse for seven weeks with the temperature maintained at 23°C. During this time counts of the

nodules were made at two-day intervals without removing the plants. At the final harvesting, nodules were counted, the plants were weighed, and the ratio of weight of root to weight of shoot was estimated.

Fig. 1. Nodule formation on diploid and tetraploid varieties of red clover inoculated with different rhizobial strains.

Observations and results

Nodules began to appear on the diploid plants about twelve days after planting, at the first leaf stage. Germination of the tetraploid plants was slower, with the onset of nodulation being correspondingly later, *i.e.* about

seventeen to nineteen days. As can be seen from Fig. 1 at the end of a seven-week period the nodule numbers produced by the strains F and SA on the diploid plants exceeded those numbers on the tetraploid plants, though in both cases a hundred per cent nodulation was achieved (Table 1). The nodules on the tetraploid plants were, however, rather larger than those on the diploid plants. In both cases, nodules formed were of the pink "effective" type, containing leghaemoglobin [4 5 13]. Strain H appeared to fall into a different category, however. Only sixty per cent nodulation was achieved on the diploid plants, and on the tetraploids this was reduced to eight per cent (Table 1). In general the weights of the tetraploid plants were greater than those of the diploids, and there was also a certain degree of correlation shown between the average number of nodules per plant and the average weight (Table 1). In well-nodulated plants the ratio weight of shoot/weight of root was greater than unity, but in the poorly nodulated plants produced by strain H, and in the uninoculated controls, it was less than unity.

TABLE 1

Weights and nodule numbers of diploid and tetraploid varieties of *Trifolium pratense* inoculated with rhizobial strains of different origin.

Rhizobial strain	F		SA		H		Uninoculated control	
Chromosome No. of plant	*2n*	*4n*	*2n*	*4n*	*2n*	*4n*	*2n*	*4n*
Nodulation (%)	100	100	100	100	66	8	0	0
Average No. of nodules per plant at 7 wks	12.2	7.7	9.56	6.16	7.52	6.5	0	0
Average fresh weight per plant at 7 wks, g	0.140	0.165	0.116	0.134	0.096	0.125	0.095	0.110
Ratio of weight of shoot to weight of root at 7 wks	1.78:1	2.6:1	2.3:1	2.7:1	1.24:1	1.9:1	0.58:1	0.7:1

Discussion

From the results, it is apparent that the three rhizobial strains tested did not all respond in the same way to the tetraploid plants as they did to the diploids. Strains F and SA, which were originally V.I.E. strains, *i.e.* virulent, incursive and effective, a term used by Strong [10] to apply to rhizobial strains, gave one hundred per cent nodulation with the diploid and the tetraploid plants though the number of nodules formed was invariably smaller in the latter. It is of interest to note that the greater numbers of nodules found with these strains on the diploid plants as compared with the tetraploids at the end of seven weeks, was due to a greater *rate* of increase in nodule numbers per unit time over the period 22 to 30 days after planting. After this time, the rate of increase of nodule numbers on both diploid and tetraploids was approximately the same (Fig. 1).

This was also demonstrated by the experiments of Nillson and Rydin [6] where a similar graph showed a comparable greater rate of increase over the period 15 to 30 days after planting. This may be of considerable physiological significance.

Strain H, however, appears to fall into a different category. A plant may be classed as either responsible *or* unresponsible, to use the terms of Nutman [7]. In the case of strain H, which was not initially strongly infective with diploid *T. pratense*, producing only 66% nodulation, the change in the chromosome number of the plant has affected its responsibility *i.e.* its response to the nodule organism, and resulted in extremely poor nodulation with only 8% of the plants producing nodules.

Bonnier [3] investigated the connection between infection and polyploidy, in experiments where clover and lucerne were grown in colchicine solution, and he found that infection was more frequent, and occurred earlier, in the presence of colchicine. This could be interpreted as being due to an increase in the number of disomatic cells in the root of the legume, as such disomatic cells which are normally present in the root cortex have been shown to be the sites of rhizobial infection (Wipf [14], Wipf and Cooper [15 16]). Also, the infected cells of the nodule itself show this disomaty. This relationship is found for polyploid as well as diploid plants so that in a tetraploid host, the disomatic cells will be octaploid. Each nodule possesses a meristem, and is considered by some workers (Thimann [11], Nutman [8 9]) to be a metamorphosed lateral root, though this theory is opposed by Allen and Allen [1] on anatomical grounds.

Whatever the cause of the reduced nodule numbers on the tetraploid plants it seems apparent that there is a need for further investigation of the nodulation of polyploid legumes, since the present work also shows that with some rhizobial strains, the actual infection of tetraploid plants is depressed, as compared with diploid plants. In the light of this evidence the possibility of selecting special rhizobial strains for use as commercial legume inocula should be considered.

Summary

The effectiveness of three different strains of *Rhizobium trifolii* on a diploid and a tetraploid variety of red clover (*Trifolium pratense*) grown in aseptic mineral-agar culture was compared. It was found that with two of the rhizobial strains tested, the tetraploid plants were slower to nodulate than the diploid plants, but that a hundred per cent nodulation was obtained in both cases. With the third strain, however, a sixty-six per cent nodulation of the diploid plants was reduced to an eight per cent nodulation of the tetraploids.

In general, over a given period, fewer nodules were formed on the tetraploid plants, but these tended to be larger than on the diploids. The fresh weight of the tetraploid plants always exceeded that of the diploids, but less so in cases of poorly nodulated plants.

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J. B. Weir

Botany Department, University College of Rhodesia and Nyasaland, Salisbury, Southern Rhodesia

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LETTER TO THE EDITORS

A Simple Technique for Determining Mineralization of Carbon during Incubation of Soils treated with Organic Materials

Introduction

During studies of the decomposition of straw and composts in soils a simple method was required for determining the rate of mineralization of organic carbon during incubation of soil treated with these materials. Soil respirometers, based on electrolytic regeneration of oxygen within the apparatus to replace oxygen consumed by metabolic processes, and apparatus based on incubation in large containers followed by suction of carbon dioxide-free air through the container to determine carbon dioxide are expensive and cumbersome and require a constant-temperature room or large constant-temperature chamber for their operation (Birch and Friend[1], Greenwood and Lees [3 4], Swaby and Passey [5], Wieringa and Mogot [7]).

Preliminary experiments

It was considered that a simple method would be to incubate the treated soil in a closed test tube containing a material which would absorb carbon dioxide and release oxygen in equivalent amounts. Preliminary tests with mixtures of hydrogen peroxide and sodium hydroxide solutions contained in a small vial resting on the soil showed that this mixture released oxygen too rapidly to be of any value. Tests using barium peroxide (B.D.H., 85–90%) plus water in the vial were tried and found to be satisfactory. Barium peroxide is slightly soluble in water giving an alkaline solution which effectively absorbs carbon dioxide and at the same time gradually releases oxygen. Further tests were carried out using test tubes and vials of different sizes and varying amounts of barium peroxide and water in the vials in order to establish a definite procedure. The carbon dioxide absorbed by the barium peroxide-water was determined by transferring the vial (which was replaced by a freshly-charged vial) to the conical flask of a calcimeter of the Collins type (Collins [2]) and reacting it with an excess of 2*N* hydrochloric acid. Under these conditions no oxygen is released and the increase in volume in the calcimeter is due solely to carbon dioxide released.

The reactions which take place in the vial are probably as follows

$$BaO_2 + H_2O + CO_2 \rightarrow BaCO_3 + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow H_2O + O \quad (2)$$

$$BaO_2 + H_2O \rightarrow Ba(OH)_2 + O \quad (3)$$

From Reactions (1) and (2) it is seen that half a mole of oxygen is released for each mole of carbon dioxide absorbed, indicating that the partial pressure of oxygen in the test tube would decline with time. It was found, however, by estimation of the residual peroxide (Vogel [6]) after reaction with HCl in the calcimeter that further oxygen had been released, presumably by Reaction (3), during incubation so that in actual fact one mole of oxygen was released for each mole of carbon dioxide absorbed. Thus the partial pressure of oxygen in the test tube remains more or less constant.

The method as finally adopted is described in detail below.

Method

Ten grams air-dried, 2-mm-sieved soil is mixed intimately with a suitable amount of ground organic material (*e.g.* 1% by weight, which is equivalent to about 10 tons per acre). The mixture is placed in a 6 inch × 0.75 inch (internal diameter) test tube and water is then added slowly from a burette to bring the soil to a convenient moisture content (*e.g.* 33 or 50 per cent of the maximum water-holding capacity). 0.2 g barium peroxide is measured * into a 1-dram vial (2 inch × 0.5 inch outer diameter) followed by 1 ml water and the vial is placed in the test tube resting on the soil. The test tube is then closed with a rubber bung. For each soil type used a control is prepared containing soil without addition of organic material. This allows for carbon dioxide released from native soil organic matter. A further control test tube containing no soil but only the vial holding barium peroxide-water is also prepared. This serves as a blank to allow for carbon dioxide contained in the barium peroxide initially and in the air of the test tube. The test tubes are placed in an incubator in a rack at an angle of about 20° from the horizontal. This increases the surface area of the alkaline solution in the vial and promotes absorption of carbon dioxide. Once each day the test tubes are shaken gently in an upright position for a few seconds to break the "scum" of barium carbonate which forms on the surface in the vials. Periodically (every 2 to 3 days during the early stages of incubation extending to 3 weeks or more during the later stages) the vial from each test tube is removed, replaced by a freshly charged vial, and its carbonate content determined in the calcimeter by reaction with 2*N* hydrochloric acid. The vials are supplied with polythene stoppers, so they may be filled at leisure and left stoppered until required. The amount of barium peroxide used (0.2 g) can cope with the release of up to about 12 mg carbon dioxide per 24 hours without oxygen becoming limiting and

* A calibrated glass tube may be used for this operation. Dry barium peroxide has little affinity for carbon dioxide, so that no precautions need be taken to exclude carbon dioxide during this process.

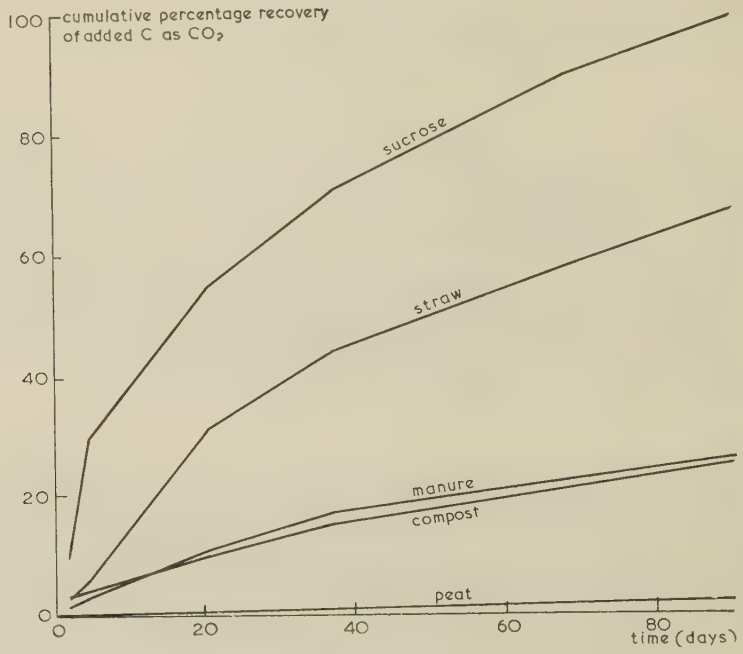

Fig. 1 Mineralization of carbon in soil treated with 1% organic materials during incubation at 28° for 90 days.

can absorb theoretically a maximum of about 50 mg carbon dioxide. It is advisable, however, to replace the vial with a freshly charged one before half the barium peroxide has reacted.

Fig. 1 illustrates the results obtained when the method was applied to determining the rate of mineralization of carbon from 10 g of a sandy soil (pH 6.2) containing 1 per cent each of added sucrose, straw, composted straw, rotted manure, and peat. Water was added to 33 per cent of the maximum water-holding capacity and incubation was carried out at 28°. Two mg NO_3-N, as potassium nitrate, was supplied initially to each soil to ensure that nitrogen was not limiting microbial activity. Results are presented as percentage cumulative recovery of added carbon as carbon dioxide.

The method described in this paper for determining mineralization of carbon is probably not as elegant or precise as are the respirometer methods and requires somewhat more manpower for its operation. However, its ability to deal with a large number of treatments in a small space and the fact that cheap and simple apparatus is used may make it of interest to other workers. Any other suitable method may be used for determining the carbonate content of the material in the vials, but the author considers that the calcimeter method is very satisfactory in that the contents of 20 tubes may be analysed in about 3 hours.

Summary

A simple technique is described for determining the rate of mineralization of carbon (carbon dioxide release) during incubation of soils treated with organic materials. The moistened mixture of soil and organic material is contained in a closed test tube and a small vial containing barium peroxide and water rests on the soil. This mixture absorbs carbon dioxide and releases oxygen in approximately equivalent amounts. The vial is removed periodically, replaced with a freshly charged vial, and its carbonate content determined in a calcimeter. Typical results obtained using a number of organic materials are presented.

A. H. Cornfield
Imperial College, London, S.W.7., England

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LETTER TO THE EDITORS

On the Rôle of Toluene in the Measurement of the Activity of Soil Enzymes.

Toluene has been used in studies of soil enzymes as an antiseptic or plasmolytic agent. Its rôle is:

1) to stop further synthesis of enzymes by living cells,
2) to prevent the assimilation of products of an enzymatic reaction, and
3) to liberate the intracellular enzymes.

Some authors hold that toluene does not fulfil these requirements. Claus and Mechsner [1] presented very convincing data, obtained by the plating method, which demonstrated that multiplication of micro-organisms took place during incubation of the soil sample with toluene and substrate. On these findings we should not use toluene in determinations of soil enzymes. But the results of other authors complicate the problem.

McLaren, Reshetko and Huber [5] also doubted the value of toluene for investigations of soil enzymes. They sterilized the soil sample by an electron beam and demonstrated that the splitting of urea in the original non-sterile soil is of the same magnitude (in some cases even lower) as in the irradiated sample where all micro-organisms were killed (controlled by the plating method). There are two explanations: either no multiplication of micro-organisms occured in the non-sterile sample during the incubation or, and this is more probable, the microbial growth occurred but did not effect the enzymatic activity. From these data it is evident that even the non-sterile sample of soil provides valid results and, consequently, the findings obtained by the plating method do not necessarily show that toluene does not meet the three requirements stated above.

Our own results support this conclusion. The second condition mentioned above was confirmed in our prelimenary experiments before our studies of soil enzymes were started [2]. Twenty grams of air-dried soil treated with none, 2.5, and 5 ml of toluene were incubated with 10 ml of 1% glucose solution and 10 ml of water at 42°C. After 24 hours the amount of glucose was determined. In the series without any toluene no reducing compound was present. On the other hand, the amount of glucose remained unchanged with 2.5 and 5 ml of toluene.

In a similar experiment 400 μM of aspartic acid were used. After incubation (64 hours, 42°C) with toluene the original amount of amino acid was obtained on the chromatogram spot. In some cases the amount of aspartic acid was

reduced but a new spot of alanine occured. This conversion was stimulated by the addition of pyruvate. We suggest that this was due to an enzymatic reaction not involving living cells [3]. In the series without any toluene the amino acid spot disappeared within 24 hours without producing any others.

These results are confirmed by respiration experiments. 0, 0.4, and 0.6 ml of toluene and 1 ml of 20% sucrose were added to 4 g of air-dried soil in the Warburg vessel. The direct method was used for carbon dioxide estimation. In the vessels without any toluene the respiration started immediately and after 10 hours it reached such an intensity that it was beyond the range of Warburg measurements. On the other hand, no respiration was observed in the series with toluene during 48 hours incubation.

We concluded that toluene does prevent the assimilation of readily available products by the soil micro-flora when used in the quantities indicated. Care must be taken to prevent evaporation, and the layer of soil suspension and covering water must not be high otherwise the conditions on the bottom of the flask are favourable for the growth of anaerobic micro-organisms.

We can summarize that toluene should not be rejected as an antiseptic agent for investigations of soil enzymes without further experimental check. However, we do not think that the original Hofmann's method and its interpretation should be accepted. Our point of view is published in another paper [4].

J. Drobník

Laboratory of Biophysics, Faculty of Science, Charles University, Praha, ČSR

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LETTER TO THE EDITORS

Über die Zuverlässigkeit der Methoden zur Bestimmung der Aktivität von Bodenenzymen nach Ed. Hofmann

Claus und Mechsner [3] haben die in unserem Institut entwickelten Methoden zur Bestimmung der Aktivität von Bodenenzymen einer Kritik unterzogen. Anlaß hierzu gab ihre Beobachtung, daß nach Abimpfen aus der Reaktionslösung auf einen toluolfreien Nährboden Entwicklung von Bakterien eingetreten ist. Diese Kritik ist u.E. unberechtigt.

1 Mit den Methoden, welche wir zuletzt beschrieben und empfohlen haben [12], erfassen wir das Ausmaß der Hydrolyse verschiedener Substrate (Kohlenhydrate, Glykoside, Harnstoff und Eiweiß). Als Maßstab für die Aktivität der Enzyme in einer Bodenprobe dient die Menge der entstandenen Spaltprodukte. Diese letzteren werden mit einfachen chemischen Methoden bestimmt. Eine Nachprüfung mit solchen Methoden zeigt, daß in Gegenwart von Toluol keine weitere Zersetzung der Substrate und der Hydrolysenprodukte erfolgt. Wohl aber ist dies bei Abwesenheit von Toluol der Fall. Diese Beobachtung entspricht ganz den jahrzehntealten Erfahrungen der Enzymologen und Bakteriologen [1]. Sie beweist andererseits wie unzuverlässig eine Bestimmung von Keimzahlen in Bodenproben nach dem Koch'schen Plattenverfahren ist, besonders dann, wenn man damit irgendwelche Aussagen über die Leistungen der so ermittelten Bodenflora begründen will.

2 Wir betrachten es als einen *Vorzug unserer Methoden*, daß sie *kein steriles Arbeiten* und die damit verbundenen umständlichen Maßnahmen erfordern, sondern in einfachster Weise über bestimmte Leistungen biologischer und biochemischer Natur Auskunft geben.

3 Während unsere Methoden [12] leicht reproduzierbar sind und sehr gut übereinstimmende Werte ergeben, werden mit dem Koch'schen Plattenverfahren nur etwa ein Tausendstel der im Boden wirklich vorhandenen Keime erfaßt, wie die neueren und modernen Untersuchungen von Strugger [11] und Burrichter [2] beweisen. Schließlich können alle Keimzahlen nichts über die mögliche oder wirklich vorhandene Umsatzleistung der so bestimmten Mikroorganismen aussagen, umsomehr als sie auch die ruhenden Formen (Sporen u.a.m.) erfassen.

Deshalb halten wir die von Claus und Mechsner ermittelten Keimzahlen als wenig geeignet, exakte biochemische Methoden zu kritisieren.

4 Seit unsere Methoden bewiesen haben, daß steriles Arbeiten nicht nötig ist, verwenden wir lufttrockene Proben [4]. In ihnen dürften „vegetative"

Formen von Bakterien kaum noch vorhanden sein, sondern nur Sporen oder andere Dauerformen, welche zu irgendwelchen Umsetzungen im natürlichen Boden, wie auch in unseren Versuchen, nicht befähigt sind. Bezüglich der Wirkung einer Trocknung an der Luft und eines Toluolzusatzes auf die Keimzahlen, kann auf Pochon und De Barjac [9] verwiesen werden. Auch das Zitat von Waksman und Starkey dürfte falsch ausgewertet sein. Wir empfehlen den Zusatz einer ausreichenden Menge von Toluol und öfteres Durchschütteln, damit die Reaktionslösung ständig Toluol enthält.

5 Nach mehrjährigen Untersuchungen haben wir über Herkunft, Form und Wirksamkeit der im Boden vorhandenen Enzyme berichtet [5 6]. Zufolge der meist hochmolekularen Natur der in den Boden gelangenden organischen Stoffe müssen die Mikroorganismen schon während ihrer Lebenszeit laufend extracellulare Enzyme nach außen und in den Boden absondern. Ein weiterer Anteil wird bei ihrer Autolyse nach dem Absterben frei (Rippel [10]). Diese Enzyme werden von den Bodenkolloiden, besonders den Tonen adsorbiert und auf diese Weise längere Zeit wirksam erhalten [8]. So entsteht ein Enzymspiegel im Boden, der durch die laufende Nachlieferung der Mikroorganismen aufgebaut, vermehrt oder erhalten wird. An seiner absoluten Höhe haben jedoch die Enzyme, welche zur Zeit der Probenahme in den gerade lebenden Mikroorganismen vorhanden sind, nur einen geringen Anteil [4 5].

6 Über die Aufgaben eines Zusatzes von Toluol bei Enzymversuchen berichten die einschlägigen Handbücher der Enzymchemie von Bamann-Myrbäck [1]. Es sei auch die Auffassung von A. Rippel [10] (S. 91) wiedergegeben: „Derartige Stoffe setzt man dann einer Enzymlösung zu, wenn man die Lebenstätigkeit und Entwicklung von Organismen unterdrücken, also nur eine enzymatische Wirkung erzielen will".

Nahezu alle Forscher, auch solche, denen unsere Arbeitsweise nicht als Grundlage gedient zu haben scheint, wählen bei Enzymuntersuchungen in Bodenproben Toluol: Zur Bestimmung von Saccharase Kuprewitsch, Vlaszuk *et al.*, Kacneljson und Ersov; von Amylase Drobnik; von Cellulase Markusne; von Xylanase Sørensen; von Phosphatase Rogers, Kroll und Kramer; von Phytase Jackman und Black; von Urease Rotini, Kuprewitsch, Conrad, Drobnik; von Proteasen Kacneljson und Ersov, Vlaszjuk *u.a.* (cit. bei [7]).

In allen diesen Fällen wurde die Wirkung von Toluol als ausreichend befunden, um hydrolytische Enzyme wenig oder gar nicht, Oxydasen hingegen vollständig zu hemmen [7].

7 Es ist bekannt, daß bei Abimpfen aus partiell sterilisierten Substraten und zwar aus den Toluol-resistenten Sporen sich oft sehr rasch eine Flora entwickelt, welche arten- und leistungsmäßig mit der ursprünglich vorhandenen nicht oder nur teilweise identisch ist. Außerdem sind solche Sporen sowohl in der betreffenden Bodenprobe, wie auch im Enzymversuch ohne Leistung und deshalb ohne Bedeutung für den biologischen Umsatz. Es darf auch diesbezüglich auf Pochon und De Barjac [9] verwiesen werden (S. 565).

Obwohl wir also die Kritik von Claus und Mechsner als unberechtigt bezeichnen müssen, werden wir gelegentlich der Veröffentlichung unserer

Untersuchungen aus den letzten Jahren noch näher auf diese Probleme eingehen, um alle Unklarheiten zu beseitigen. Wir schließen mit den Befunden bei der enzymatischen Hydrolyse von Saccharose, Stärke und Harnstoff durch Bodenproben sowohl mit wie auch ohne Toluolzusatz.

TABELLE 1

Versuche mit *Saccharase*. Substrat Saccharose. Hydrolyseprodukte = Glukose + Fruktose			
Toluol	Puffer	Zucker als Saccharose berechnet. Pro 20 ml Ansatz	
		Bei Versuchsbeginn = ø 404.5 mg	
		nach 24 Stunden bei 37° C	
		mg	%
+	+	405.7	100.3
+	—	402.8	99.6
—	+	402.8	99.6
—	—	393.2	97.2

TABELLE 2

Versuche mit *Amylase*. Substrat Stärke. Hydrolyseprodukt = Glukose			
Toluol	Puffer	Stärke als Glukose berechnet. Pro 20 ml Ansatz	
		Bei Versuchsbeginn = ø 65.4 mg	
		nach 96 Stunden bei 37° C	
		mg	%
+	+	64.6	98.8
+	—	65.5	100.2
—	+	64.7	98.9
—	—	50.2	76.8

TABELLE 3

Versuche mit *Urease*. Substrat Harnstoff. Hydrolyseprodukt = Ammoniak			
Toluol	Puffer	N im Gesamtvolumen *	
		Bei Versuchsbeginn = ø 478.6 mg	
		nach 48 Stunden bei 37° C	
		mg	%
+	+	478.5	100.0
+	—	476.8	99.6
—	+	294.2	61.5
—	—	163.0	34.1
* Harnstoff + NH_3 als N berechnet.			

Die Befunde zeigen, daß innerhalb der Analysenfehler, bei Toluolzusatz keine Verluste an Substrat und an Hydrolysenprodukten erfolgen. Bei Abwesenheit von Toluol entstehen besonders in den Versuchen mit Stärke und Harnstoff größere Verluste. Diese sind am geringsten mit Rohrzucker als Substrat. Von ihm ist bekannt, daß er schon von sich aus die Tätigkeit der meisten Bakterien hemmt, sobald er in einer gewissen Konzentration zugegen ist. Ebenso kann ein Pufferzusatz die Tätigkeit vieler Mikroorganismen bis zu einem gewissen Ausmaß verhindern bzw. herabsetzen.

Ed. Hofmann und Gg. Hoffmann

Institut für Agrikulturchemie, Weihenstephan, d. Techn. Hochschule, München

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